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**EXERCISE ASSOCIATED FACTORS AFFECTING
CARDIOVASCULAR HEALTH AND FUNCTION:
FROM MYOCYTE SIGNALING TO GENETIC
VARIATIONS**

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active girls.
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EXERCISE ASSOCIATED FACTORS AFFECTING CARDIOVASCULAR HEALTH AND FUNCTION: FROM MYOCYTE SIGNALING TO GENETIC VARIATIONS

Introduction

The National Cholesterol Education Program's Adult Treatment Panel III report (ATP III)¹ identified the metabolic syndrome (MS) as a multiplex risk factor for cardiovascular disease (CVD) that is deserving of more clinical attention. The MS is characterized by a group of metabolic risk factors in one person. They include:

abdominal obesity, atherogenic dyslipidemia, elevated blood pressure, insulin resistance or glucose intolerance, prothrombotic state, proinflammatory state [1]. A meta-Analysis study demonstrates that the MS is associated with a 2-fold increase in cardiovascular outcomes and a 1.5-fold increase in all-cause mortality [2]. The prevalence of the MS across the U.S. population age 20-70 years was reported to be 23.7%, [range from 6.7-43.5%] with an increase in prevalence correlated with age [3]. A similar trend has also been reported in Europe with a prevalence rate of 37% across a population aged 30-89 years, [range from 10.3-58.4%] with an increase in prevalence correlated with age [4].

It is widely recognized that the most successful way of treated the MS is its prevention, with the most effective way of prevention being adopting a healthy lifestyle. Non excessive eating and regular physical activity are the factors of healthy living that have the strongest correlation to the prevention of the MS and subsequently CVD and heart failure [5]. Heart failure represent a major cause of morbidity and mortality in industrialized countries.

The preventative mechanism of physical activity with regards to MS and CVD does not solely depend on the prevention of caloric overconsumption by increasing

caloric expenditure, but also causes various cellular adaptations that have been demonstrated to be cardioprotective. The exact mechanisms of the exercise induced adaptations is not lucid, but recent studies have delineated two means of signaling by which the adaptations occur (1) substrate availability signaling (metabolic stress) (2) hormone-receptor signaling. We have decided to specifically investigate two metabolic signaling enzymes [AMP-activated kinase (AMPK) and Sirtuin 1(SIRT1)] and two hormones [Adiponectin and Adrenergic stimulation] that are at the moment frequently investigated as potential targets to cardiac protection.

AMP-activated kinase (AMPK)

5'Adenosine mono-phosphate protein kinase is composed of three subunits: alpha, beta, gamma. The alpha subunit is the site of phosphorylation (Thr 172) that occurs to activate the AMPK complex. This subunit can only be phosphorylated if the complex has been allosterically activated, that occurs on the gamma subunit.

The beta subunit of the complex contains a glycogen binding site, and is thought to be the subunit that holds the other two subunits together. The gamma subunit contains two Bateman domains which is where AMP binds [6].

AMP's binding to the gamma subunit allosterically promotes the phosphorylation of the complex at the alpha subunit by an upstream kinase: those that have been recently identified are: a complex between the tumor suppressor protein LKB1 and accessory subunits termed STRAD and MO25 [7] and CAMKKbeta, found to be the upstream kinase in the brain, thymus, T cells, and tumor cells. Apart from promoting phosphorylation AMP binding also makes the AMPK complex a worse substrate for protein phosphatases that dephosphorylate the alpha subunit (Thr 172). (Figure 1)

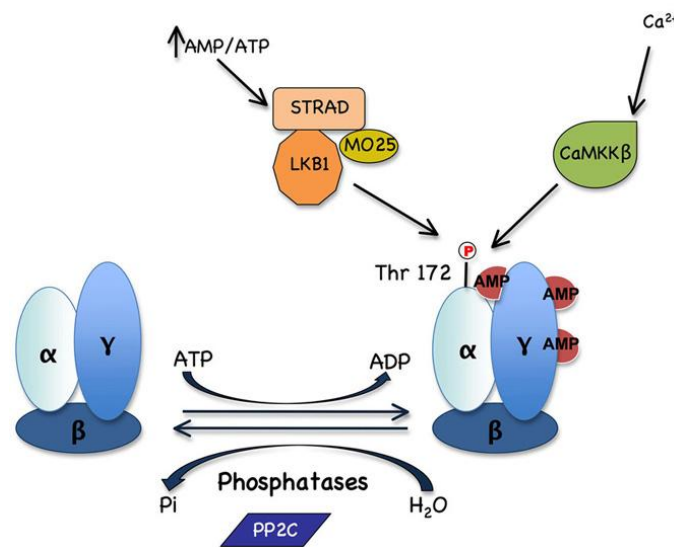


Figure 1

Biochemical studies from the end of last century were showing that gene encoding for Adenosine mono-phosphate protein kinase was present in all eukaryotic species, vertebrates and invertebrates [8] Following, this same gene coding was found to be present and involved in the yeast *Saccharomycyes Cervisiae* response to glucose starvation [9] as well as during the darkness period of green plants, when without the sun, therefore lack of photosynthesis, are unable to produce glucose. AMPK, a trimmer with two AMP binding sites, has been termed as the cell's "energy gage", sensing any slight change increase in cellular AMP, causes an immediate activation of various cascades mechanism: activation of energy producing pathways, and blocking energy depletion pathways (protein synthesis). (Figure 2).

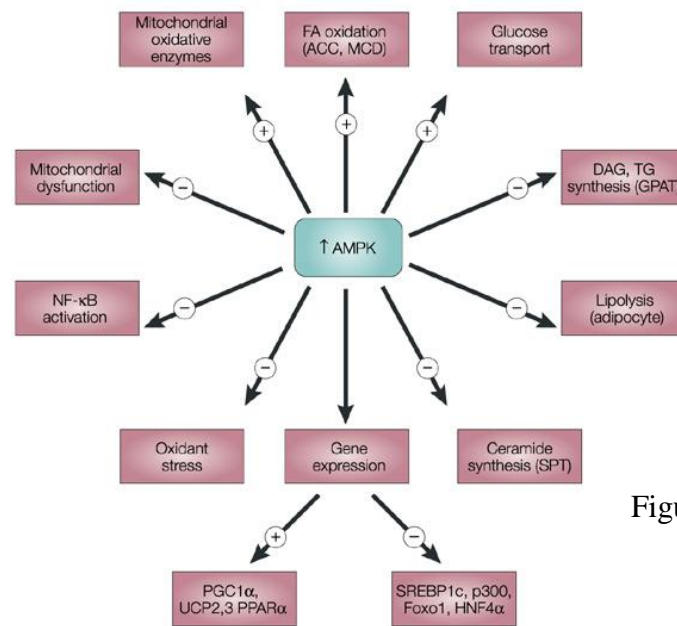


Figure 2

After the discovery of AMPK's key role in the muscular adaptations that occur following exercise [10], research began to unfold in the cardiovascular field, and is now recognized as playing a critical physiological role in the cardiovascular system. Increasing evidence suggest that AMPK might also function as a sensor by responding to oxidative stress. Mostly importantly, AMPK modulates endogenous antioxidant gene expression and/or suppress the production of oxidants. AMPK promotes cardiovascular homeostasis by ensuring an optimum redox balance on the heart and vascular tissues. Dysfunctional AMPK is thought to underlie several cardiovascular pathologies [11].

Sirtuin 1

Sirt1 is the human homolog of Sir2, part of the family of NAD⁺ dependent deacetylase. Its discovery in 2000 by Leonard Guarente first demonstrated that Sir2 controlled the longevity of yeast, which following lead to a hype in research exploring this area in search for the human homolog [12]. To the pleasant surprise of many, the human homolog of Sir2, Sirt1, has revealed to have more positive effects than expected, aside from effecting the longevity of an organism, it plays a critical role in the regulation of

metabolism in response to changes in nutritional availability in multiple tissues. (Figure 3). [13].

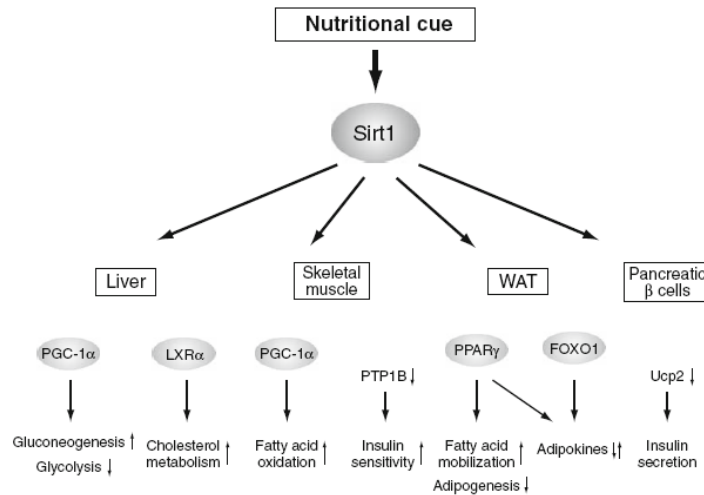


Figure 3

SIRT1 is located in the nucleus of a cell. It activates downstream pathways by deacetylating its target proteins' lysine and placing the acetyl on the NAD molecule, to then form 2-o acetyl ADP ribose and nicotinamide(Fig 4) , the substrate then used in NAD⁺ biosynthesis (Fig 5) . [14].

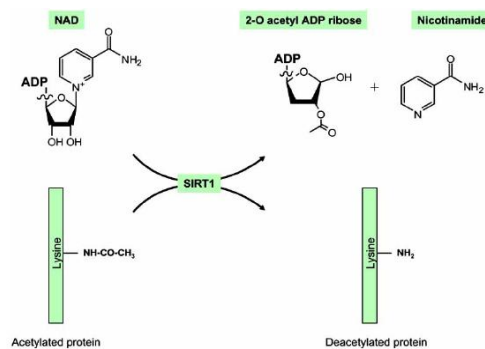


Figure 4

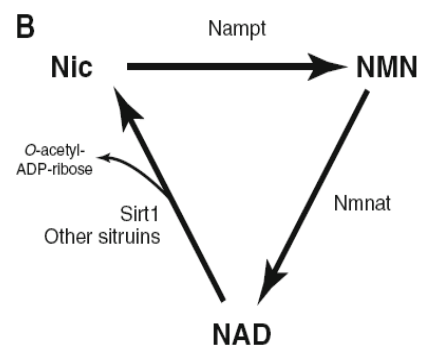


Figure 5

SIRT1 is said to receive its metabolic status information via NAD availability. NAD is found in the nucleus as the reducer, proton donor, NADH, a crucial part of cellular

metabolism: glycolysis, citric acid cycle, and oxidation phosphorylation. As the rate of metabolism increases, there is an increase in the presence of NAD⁺ and the ratio NAD⁺/NADH increases. The increase in NAD⁺ availability not only activates SIRT1, but its presence alone allows for it to deacetylate a downstream protein. SIRT1 can deacetylate both acetylated histone proteins, including acetylated histone residues H3K56 [15], H4K16, and H3K9 [16,17], and non-histone targets such as p53, FOXO-family transcription factors, and PGC-1α [18, 19, 20].

It has been recently demonstrated that only after 4 days of high intensity interval training in humans not only increases mitochondrial biogenesis (well-known), but also that the increase in biogenesis is also coupled with an increase PGC-1α protein and increase SIRT1 activity but a decrease in SIRT1 protein [21]. When comparing aging effects with exercise effects in mice heart, it was concluded that exercise training, which significantly increases SIRT1 activity, could counteract age-related systems impairment. [22].

Adiponectin

Adiponectin is a 247-amino acid peptide with a collagenous domain at the n-terminus and a globular domain that shares substantial homology with the subunits of complement factor C1q [23]. Adiponectin is mainly secreted from adipose tissue, in humans, however, its expression was also found in bone marrow, osteoblasts, fetal tissue, myocytes, cardiomyocytes and salivary gland epithelial cells [23]. What makes adiponectin a particularly interesting adipokine is that population studies show that while the majority of secreted hormones increase as fat mass increases, adiponectin, on the other hand is inversely related with fat mass, more specifically with visceral fat, and its decrease is independently associated to the metabolic syndrome [24,25]. It has

been found that adiponectin inhibits monocyte adhesion to endothelial cells , macrophage transformation to foam cells , and vascular smooth muscle cell proliferation in vitro. In vivo, forced adiponectin expression reduces atherosclerotic lesions in a mouse model of atherosclerosis , whereas adiponectin-deficient mice exhibit excessive vascular remodeling response to acute injury and diet-induced insulin resistance. Therefore, adiponectin has gained considerable attention because of its antidiabetic, antiatherogenic and anti-inflammatory properties [26].

The discovery of the adiponectin receptors (AdipoR1, AdipoR2 and T-cadherin) as well as their extensive distribution in peripheral tissues and organs further suggests that adiponectin exerts pleiotropic effects on whole-body metabolism [23] (Figure 6). Both receptors have homology to G-protein coupled receptors, and both effect the downstream kinase of AMPK, however mouse knockout studies linked AdipoR1 more closely to the activation of the AMPK pathway, whereas AdipoR2 is more tightly involved in the PPAR α pathway.

The regulation of adiponectin expression has yet to be fully determined. Analysis of the promoter region of the human adiponectin gene revealed several regulatory elements. Recent studies show that SIRT1 can up-regulate adiponectin gene expression by enhancing the formation of a FOXO1-C/EBP α transcription complex, which is recruited to the adiponectin promoter [23].

In relation to physical activity it was demonstrated that in humans, vigorous exercise increased serum adiponectin when compared to their sedentary counterparts independent of weight [27]. Not only is serum adiponectin increased but also adiponectin receptors increases following training in skeletal muscle [28].

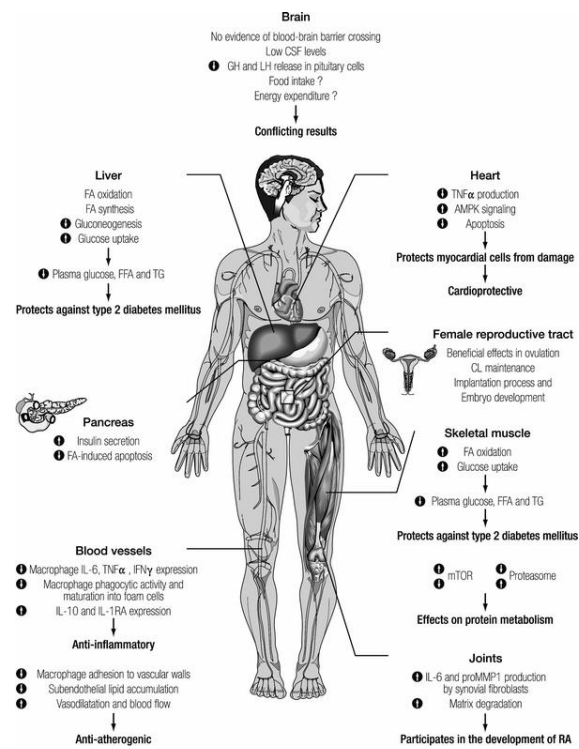


Figure 6

Following are presented three scientific articles that have been published resulting from last three years of doctoral research, each one having to do with a different aspect of the proteins described above.

CHAPTER 1: UPREGULATION OF SIRT1 DEACETYLASE IN PHENYLEPHRINE-TREATED CARDIOMYOBLASTS

INTRODUCTION

Disparate mechanisms of cardiac stress invoke a common pathway of adaptive myocyte hypertrophy which progresses into impaired systolic function and dilated, thinned myocardium, and finally heart failure, which is presently the leading cause of hospitalization and death in developed countries [1]. Recently, members of the sirtuin family, specifically SIRT1, have become a focus in cardiovascular research [2,3]. SIRT1 is an ubiquitous, nuclear and cytoplasmic NAD⁺ dependent deacetylase that deacetylates both acetylated histone and non-histone targets [4]. SIRT1, thought to be implicated in the control of longevity and aging, has an important role in metabolic regulation and receives its metabolic status information via NAD⁺ availability [2]. Various studies have shown SIRT1 activation as protective against cellular stress. In cardiac cellular models, SIRT1 has been found to be protective against hypertrophic, oxidative, and metabolic stress resulting in increased cell survival [5-9]. The link between SIRT1 and cardiac hypertrophy is however complex and not fully understood. In the whole animal, cardiac hypertrophy is associated with increased SIRT1 expression [5,10,11] whereas, on the other hand, SIRT1 overexpression or activation by resveratrol results in the inhibition of hypertrophy [12, 13]. Different effects can also be obtained depending on the extent of SIRT1 overexpression [3,14]

Another protein involved in the response to cellular stress is AMP-activated protein kinase (AMPK), a key enzyme that regulates cellular energy state, growth, and many other cellular functions [15]. Analogous to SIRT1, the role of AMPK in cardiac

hypertrophy is complex, but it is however acknowledged that AMPK has an anti-hypertrophic role, because when activated it inhibits ATP consuming pathways such as protein synthesis [16]. Being both, SIRT1 and AMPK, enzymes that regulate metabolism, it was not surprising to see that SIRT1 can activate AMPK, whereas AMPK in turn can activate SIRT1 [17]. Since both SIRT1 and AMPK are implicated as being protective against cardiac hypertrophy and have been suggested as pharmaceutical targets [18,19], understanding their response under hypertrophic stress is of importance. With this in aim, we have studied in H9c2 cardiac myoblasts, the response of SIRT1 to phenylephrine, recognized to induce a hypertrophic process in this cardiac cell model [20,21].

MATERIALS AND METHODS

Cell culture and treatment

H9c2 cardiomyoblasts (embryonic rat heart derived cells) were cultured in Dulbecco's modified Eagle's medium (DMEM, Celbio) supplemented with 10% heat inactivated fetal calf serum (FCS), 5% glutamine and antibiotics. Before the treatments, the cells were serum starved for 18 h in medium containing 1% FCS. All tested compounds were dissolved in phosphate-buffered saline (PBS) or DMSO at a 1000x concentration. Control cells received the corresponding volume of the vehicle.

Cell viability was determined by trypan blue exclusion. Samples were done in triplicate, and at least 10 fields were counted for each sample. Cell survival was calculated as the percentage of living viable cells in treated samples in respect to the number of viable cells in control samples.

Western Blotting

H9c2 cells were collected in lysis buffer (5 mM dithiothreitol, 2 mM EDTA, 0.1% CHAPS, 0.1% Triton X-100, and protease inhibitors in 20 mM HEPES pH 7.5) and subjected to two cycles of freeze-thawing. The homogenate was then centrifuged at $15,000 \times g$ for 15 min and the supernatant, diluted in loading buffer (2% SDS, 5% glycerol, 0.002% bromophenol blue, 4% β -mercaptoethanol in 0.25 M Tris-HCl, pH 6.8) and boiled for 4 min. Aliquots corresponding to 120 μ g protein were analyzed by SDS-PAGE and immunoblotting. Immunoreactive bands were visualized by chemiluminescence with the ECL reagent (Amersham). Anti-SIRT1 and anti β -actin were obtained from Santa Cruz. Antibodies against phospho-Acetyl Coenzyme A Carboxylase β (ACC) (Ser79), AMPK α 1/ α 2 and phospho-AMPK α 1/ α 2 (Thr172) were from Cell Signaling. β -Actin was used as internal control. Quantitative assay of blots was obtained by densitometry with a Fluor-S Max Multimager (Bio-Rad).

SIRT1 activity

The enzymatic deacetylase activity of SIRT1 was assayed using an immune-complex fluorometric assay. SIRT1 was immunoprecipitated from H9c2 soluble extracts (0.5 mg of protein) [22], and the final SIRT1-immune-complex was resuspended in 0.16 ml of assay buffer (5 mM dithiothreitol, 0.1 M NaCl, 0.1% Triton X-100, 20% glycerol, 20 mM Tris/HCl pH 7.6). The sample divided into four 40 μ l aliquots that were separately assayed (in presence or absence of NAD^+) in a final volume of 50 μ l containing 100 μ M NAD^+ and 100 μ M of the acetylated tetrapeptide RHKKAc-AMC [23] purchased from LifeTein, South Plainfield, NJ. This peptide is based on the amino acid sequence of human p53 close to Lys382. The reaction was incubated for 90 min at 37°C, afterward

50 μ l of stop solution (40 mM nicotinamide, 10 mg/ml trypsin, 0.2 M NaCl in 0.1 M Tris pH 8) were added. After further 20 min at 37 °C, 2.5 ml of water were added to each tube and fluorescence was read in a Perkin Elmer fluorescence spectrometer (ex 370, em 450). SIRT1 activity was calculated from the fluorescence developed in the complete assay, after subtraction of the fluorescence measured in absence of NAD^+ . Preliminary experiments to optimize assay linearity and to calculate the recovery of SIRT1 activity in the whole procedure, were done by adding to extracts a known amount of recombinant SIRT1 protein (Sigma-Aldrich).

Cell metabolites

Acid-soluble metabolites were extracted in ice cold 0, 3M perchloric acid and measured by HPLC after extract neutralization with buffered 3M KOH. The cellular content of ATP and other nucleotides was determined after conversion into fluorescent etheno-derivatives [24]. NAD^+ and NADH were directly determined in the neutralized extract by detection at 260 nm after separation [25].

RNA interference

The sequences of siRNAs directed against rat SIRT1 and the catalytic subunits of AMPK (Obtained from Sigma-Genosys) were as follows: for SIRT1, 5'-CACCUGAGUUGGAUGAUAU-3'; for $\text{AMPK}\alpha 1$, 5'-CUUAUUGGAUUUCCGAAGUTT-3'; for $\text{AMPK}\alpha 2$, 5'-GACAUUAUGGCGGAGGUGUTT-3'. Control siRNA-A was purchased from Santa Cruz Biotechnology. Cells at 50 % of confluence were transfected with a final concentration of 100 nM siRNA duplex for 24 h by Transfection reagent (Santa Cruz)

according to manufacturer's instructions. 24 hours after transfection, cells were then treated with the designated drugs.

Data analysis

All the experiments shown were performed independently at least three times with comparable results. The blots are representative of results obtained in multiple experiments. All the data presented in graphs are expressed as means \pm S.E. of the mean of the indicated numbers of determinations. Comparison between two groups were done by Student's unpaired two-tailed *t* test. Differences were considered as significant when $P < 0.05$.

RESULTS

H9c2 heart cells represent a useful model in which molecular event associated to hypertrophy can be studied [26]. In these cells, the adrenergic effector phenylephrine causes a hypertrophic response similar to that evoked in primary cardiomyocytes and in the whole heart [20,21]. Since sirtuins are associated to the cell stress response, the hypothesis that phenylephrine could influence SIRT1 was tested. Analysis of soluble extracts prepared from whole H9c2 cell lysates showed increased SIRT1 protein levels in cells treated with 10 μ g/ml phenylephrine for 24-48 h with respect to control cells (Fig. 1A). The increase of SIRT1 at 24 h observed in several independent experiments ranged from 1.6 to 3.5 times as determined by densitometric analysis of bands obtained by Western blotting. Fig. 1B shows that even the deacetylase activity of SIRT1 in soluble extracts was significantly increased following 24 h of treatments with phenylephrine. This activity was defined as the enzymatic activity immunoprecipitable by SIRT1 monoclonal antibody that deacetylates an acetylated-peptide substrate in the

presence of NAD^+ . The content of SIRT1 protein was also increased by the physiological adrenergic effector norepinephrine (10 $\mu\text{g/ml}$), whereas pretreatment with the non-specific alpha-receptor antagonist phentolamine partially inhibited the phenylephrine-induced increase in SIRT1 expression (Fig. 1C).

Interestingly, it has been reported that phenylephrine rapidly stimulates the phosphorylation of the catalytic α -subunit of AMPK within a few minutes in H9c2 cells [27]. The experiment depicted in Fig. 2A confirms an increase in AMPK- α (Thr172) phosphorylation following treatment with phenylephrine (10 $\mu\text{g/ml}$), and shows that this is not a strictly transient event, being detectable 1h and 2 h after the treatment. To determine whether AMPK activity was linked to the augmented expression of SIRT1, a pharmacological approach was firstly used (Fig. 2B). When H9c2 cardiomyoblasts were exposed for 24 hours to phenylephrine in the presence of AMPK inhibitors AraA (0.5 mM) [28] or STO-609 (1 μM) [29], the increase in SIRT1 levels was not evident. On the other hand, the AMPK activator AICAR (4 mM) significantly increased the effect of phenylephrine on SIRT1 level. The effects of the aforementioned AMPK modulators on SIRT1 was very similar to that caused on the phosphorylation of ACC (Ser79), a physiological substrate of AMPK, suggesting an association between AMPK activity and SIRT upregulation by phenylephrine. To support this suggestion, a siRNA approach was used. The cells were treated with two siRNAs respectively targeted against the $\alpha 1$ and $\alpha 2$ catalytic subunits of AMPK in order to knockdown AMPK activity, or with a control siRNA of random sequence without any target- The cardiomyoblasts were then exposed for 24 hours to phenylephrine. Fig. 2C shows that inhibition of AMPK $\alpha 1/\alpha 2$ expression by siRNA caused a significant degree of reduction

in SIRT1 content in phenylephrine-treated cells. Taken together, these results strongly suggest that upregulation of SIRT1 by phenylephrine depends on AMPK activity.

The sensing of cellular metabolic stress by AMPK and SIRT1 is mediated through changes in the levels of adenine nucleotide and NAD^+ respectively. However, we were not able to detect any significant change in the cellular content of AMP, ADP, ATP, NADH and NAD^+ in cells treated with phenylephrine for 1, 4 or 24 hours with respect to control cells (data not shown).

In order to evaluate whether MAPK or Akt pathways, associated to adrenergic stimulation of heart cells, could be involved together with AMPK in SIRT1 activation, we tested the effect of pharmacologic inhibitors in phenylephrine-treated H9c2 cells. The inhibitors of the MAPK cascade U0126 (10 μM) and PD98059 (50 μM) were without any effect, whereas the PI3K/Akt pathway inhibitor LY294002 (20 μM) reduced SIRT1 activation (Fig. 2D).

In order to define whether SIRT1 could exert a protective effect in H9c2 cells exposed to hypertrophic stress, the cells were incubated 72 h with increasing doses of phenylephrine in the presence of the SIRT1 inhibitor sirtinol (20 μM). Fig. 3A shows that at the used concentration, sirtinol did not elicit toxic effect in untreated cells. On the other hand, when SIRT1 was inhibited by sirtinol in phenylephrine-treated cells, the cell survival was significantly decreased, suggesting a protective action of SIRT1 in stressed cells. To confirm this suggestion, SIRT1 expression was knocked-down by siRNA. The levels of SIRT1 was significantly decreased in cells treated with SIRT1-siRNA, compared to untreated cells and to cells treated with control siRNA (Fig. 3B). In control cells, treatment with either control siRNA or SIRT1-siRNA caused a similar decrease of viability, probably caused by unspecific toxicity of the transfection procedure.

Conversely, when the cardiomyoblasts were exposed to phenylephrine, cell death was largely increased in SIRT1 knocked-down cells, where cell survival was reduced to less than 50%, a value significantly lower with respect to that determined in cells pretreated with control siRNA. Altogether, these data indicate that SIRT1 is important to support survival in cardiomyoblasts treated with phenylephrine.

DISCUSSION

SIRT1 plays a role in biological processes such as longevity, apoptosis, and metabolic stress resistance by deacetylating histones H4 and H3 and other proteins including p53, Forkhead box transcription factors, nuclear factor kappa B, and peroxisome proliferator activated gamma coactivator 1a [2-4]. Since their discovery, sirtuins have been linked with metabolic and cardiovascular diseases, including heart hypertrophy [2,3]. SIRT1, the most studied sirtuin, was first investigated as the mechanism behind the increase in lifespan due to caloric restriction, but SIRT1 enzymatic activity has also been linked to the known beneficial adaptations seen with aerobic physical activity [30] that represent a valid treatment for metabolic and cardiovascular diseases [31]. Results from this study can suggest that the adaptive signaling leading to SIRT1 upregulation that occurs during exercise (including physiological cardiac hypertrophy) or during the development of pathological cardiac hypertrophy can also be in response to the increase in adrenergic stimulation.

We have shown that adrenergic stimulation causes an increase in SIRT1 protein and activity in cardiomyoblasts, that is protective against the cell damage associated to hypertrophic stress. This finding is consistent with previous reports showing that SIRT1 upregulation by overexpression or resveratrol results in heart cell cytoprotection against oxidative and hypertrophic stresses [5,7,9]. Our study however, shows that the same

trigger of the hypertrophic signaling causes SIRT1 upregulation, coherently with the increased SIRT1 expression observed in some models of cardiac hypertrophy [10,11]. On the other hand, SIRT1 can also inhibit the development of hypertrophy [12,13], suggesting that phenylephrine activates simultaneously both hypertrophic programming and a self-protecting/self-regulating mechanism.

Our results also imply that the signaling axis leading to SIRT1 induction involves AMPK. Adrenergic agonists activate AMPK in several cells and tissues, including different cardiac models [27,32-34]. Regarding H9c2 cells, Xu and coworkers [27] reported a rapid increase in AMPK α Thr172 phosphorylation in response to phenylephrine. Recently, Pang et al. [35] reported that Ser485/491 is the main site of rat heart AMPK phosphorylation in response to phenylephrine. In our hands, phenylephrine stimulated H9c2 AMPK α phosphorylation detected by means of an antibody directed against phospho-Thr172. This effect could be seen for a few hours from the treatment and was associated to increased enzyme activity, as shown by the elevation in ACC phosphorylation. However, the basal degree of AMPK α (Thr172) and ACC (Ser79) phosphorylation in control cells indicate a constitutive basal level of AMPK activity, hence the exact role of AMPK activation and of AMPK phosphorylation site on SIRT1 activation remains to be defined, since our data only show that the presence of an active AMPK is required for SIRT1 upregulation.

Other studies have reported that SIRT1 activity can be mediated by AMPK [36,37]. In these studies AMPK activation was achieved by glucose restriction or pharmacological activation (AICAR, metformin, or dinitrophenol) and was associated with the increase in NAD⁺/NADH ratio. We were unable to detect any changes in NAD⁺, NADH, ATP, ADP, AMP concentrations, suggesting that adrenergic stimulation upregulates Sirt1 via

a metabolism-independent pathway; even so, AMPK seems to play an important mediating role. On the other hand, additional signaling pathways are probably involved in SIRT1 activation in response to phenylephrine. The kinase Akt is an interesting candidate, since it is activated in response to phenylephrine [38,439] and can lead to induction of proteins associated with cell survival, including SIRT1 [40]. Furthermore, Akt activity can be increased upon AMPK activation that inhibits mTOR, a downstream targets of Akt, so then preventing feedback inhibition of PI3K and Akt [41,42]. Interestingly, during cardiomyocyte response to oxidative stress, AMPK and Akt collaborate to activate GLUT4 translocation [43]. We have observed that SIRT1 upregulation by phenylephrine is abrogated by the PI3K inhibitor LY294002, supporting a role of Akt in SIRT1 regulation. Following experiments will investigate the role of Ca^{++} -activated pathways in the effect of phenylephrine on SIRT1, since IP3/ Ca^{++} signaling leads to Akt activation, whereas the Ca^{++} /CaM/CaMKK axis may activate AMPK.

In conclusion, a chronic exposure of H9c2 cardiomyoblasts to the α -adrenergic agonist phenylephrine caused an increase in SIRT1 protein expression along with its activity. SIRT1 upregulation required AMPK activity and promoted cell survival. We so then propose that the increase in SIRT1 activity and expression is an adaptive response to hypertrophic stress, suggesting that adrenergic stimulation of heart cells along with the hypertrophic programming also promotes self-protecting and self-regulating mechanisms.

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Figures

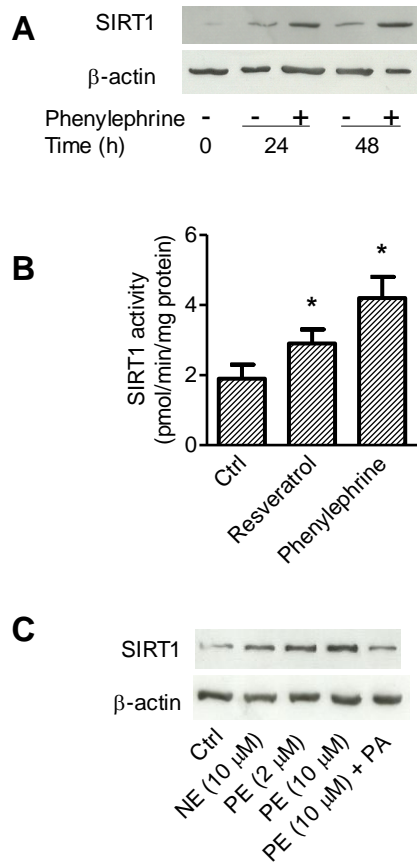


Fig.1. SIRT1 is upregulated by phenylephrine in H9c2 cardiomyoblasts. (A) The cells were incubated in the absence or presence of 10 μ M phenylephrine for the indicated time. The content of SIRT1 was then determined in cell lysates by Western blotting. (B) The deacetylase activity of SIRT1 was measured in extracts obtained from control cells and from cells incubated 24 h with 20 μ M resveratrol or 10 μ M phenylephrine. Results are means \pm SE of four determinations. *, $P < 0.05$ vs. control cells. (C) The content of SIRT1 protein was determined in lysates from cells incubated 24 h in the presence of the indicated concentration of norepinephrine (NE) or phenylephrine (PE). Phentolamine (PA) concentration was 10 μ M.

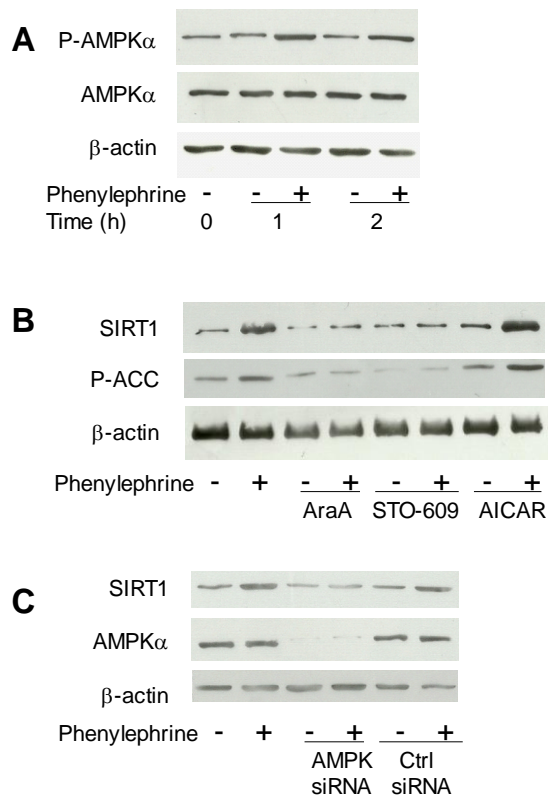


Fig. 2. AMPK involvement in SIRT1 upregulation by phenylephrine in H9c2 cardiomyoblasts. (A) The cells were incubated in the absence or presence of 10 μ M phenylephrine for the indicated time. To assay phosphorylation and amount of AMPK, cell lysates were analysed by Western blotting with antibodies against α subunits of AMPK (AMPK α) or Thr172-phosphorylated α subunits of AMPK (P-AMPK α). (B) The cells were incubated for 24 h in the absence or presence of 10 μ M phenylephrine and the indicated compounds: AraA (0.5 mM), STO-609 (1 μ M), AICAR (4 mM). The content of SIRT1 and phospho-ACC (Ser79) were assayed in cell lysates by Western blotting. (C) The amount of SIRT1 and AMPK α were determined in cell extracts obtained from cells incubated for 24 h in the absence or presence of 10 μ M phenylephrine after transfection with AMPK α 1 plus AMPK α 2 siRNAs (AMPK siRNA) or control siRNA.

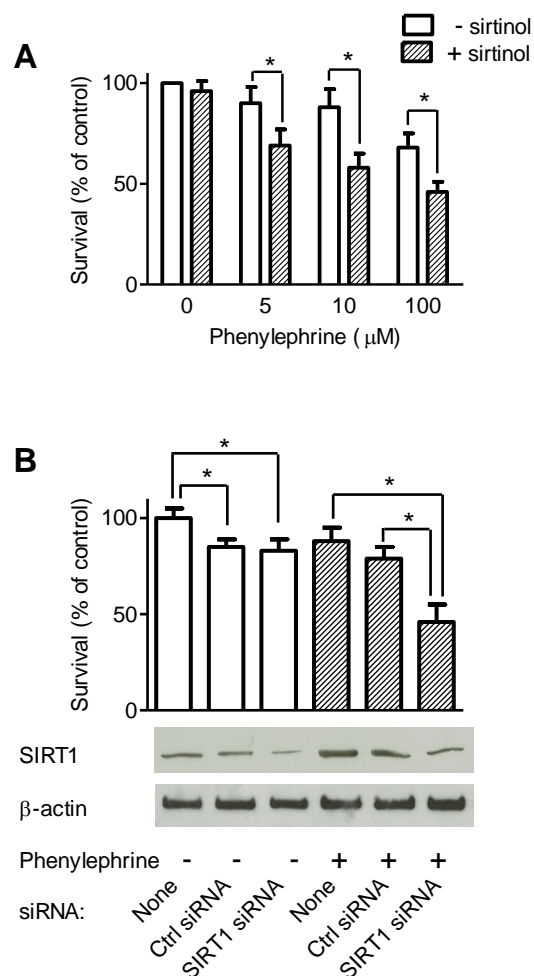


Fig. 3. SIRT1 promotes cell survival of phenylephrine-treated H9c2 cardiomyoblasts. (A) Cell viability was measured following incubation for 72 h with the indicated concentration of phenylephrine in the absence or presence of 20 μM sirtinol. Results are means \pm SE, $n=3$. (B) Untransfected cells and cells transfected with SIRT1 siRNA or unrelated control siRNA were incubated for 72 h in the absence or presence of 10 μM phenylephrine. The extent of cell death was then measured. Values are expressed as means \pm SE of triplicate determinations, *, $P < 0.05$. The blots show the effect of siRNA transfection on SIRT1 expression and are representative of three experiments.

CHAPTER 2: EVIDENCE THAT AMP-ACTIVATED PROTEIN KINASE CAN NEGATIVELY MODULATE ORNITHINE DECARBOXYLASE IN CARDIAC MYOBLASTS

INTRODUCTION

Cardiac hypertrophy and subsequent progression to heart failure represents a major cause of morbidity and mortality in industrialized countries. The defining features of cardiac hypertrophy are an increase in cardiomyocyte size, enhanced protein synthesis, and a higher degree of sarcomere organization [1]. These changes are preceded and accompanied by the re-induction of the so-called fetal cardiac gene program, characterized by a pattern of altered gene expression that mimics that observed during embryonic heart development [2]. Cardiac hypertrophy is also associated with a shift from fatty acids to glucose as energy source, an additional feature in common with the fetal heart.

A characteristic event in response to agents that induce cardiac hypertrophy is the upregulation of Ornithine decarboxylase (ODC), the key enzyme in polyamine biosynthesis. The correlation between polyamines and cardiac hypertrophy has been well established [3] and all cardiac hypertrophy inducers cause an early induction of cardiac ODC in both whole animal [4-6] and cellular models [7-9]. In the classical model of adrenergic agonist-induced hypertrophy, pharmacological inhibition of ODC results in abrogation or attenuation of the hypertrophic response [5-7,10], indicating a key role of ODC in this process, as confirmed by the marked increased in sensitivity to isoproterenol-induced hypertrophy of transgenic mice with targeted overexpression of ODC in the heart [11].

Adenosine monophosphate (AMP)-activated protein kinase (AMPK) is a key metabolic regulator that is activated in response to metabolic stress (altered energy supply and/or demand). Additionally, this enzyme can be activated by hormones that influence cellular metabolism, such as adiponectin and catecholamines [12]. The role of AMPK in cardiac hypertrophy is complex, as demonstrated by contrasting findings in literature. Many papers, reviewed in [13,14], have shown that AMPK has an anti-hypertrophic role. In this respect, genetic models of diminished heart AMPK activity show an enhanced cardiac hypertrophy [15,16] and AMPK inhibits ATP consuming pathways such as protein synthesis [17]. On the other hand, activation of AMPK in hypertrophied hearts has been observed in a number of studies, reviewed in [14,18], and could be associated to the increase energetic requirements of hypertrophic heart [19-21]. Furthermore, adrenergic agonists, classic inducers of cardiac hypertrophy either in vivo and in vitro, cause increased AMPK activation in several cell lines and tissues, including different cardiac models [22-25]. In this light, the typical ODC induction triggered by adrenergic agonists discussed above, seems to be in contrast with the well documented homeostatic role of AMPK that inhibits protein synthesis by interfering with the mTOR pathway [17, 26]. To date, nothing is known about the possible interrelation between AMPK and ODC. In the study presented below we examined the adrenergic agonist response of these two enzymes in H9c2 heart cells, a useful model in which molecular events associated to hypertrophy can be studied [27].

MATERIALS AND METHODS

Cell culture and treatment

H9c2 cardiomyoblasts (embryonic rat-heart derived cells) were cultured in Dulbecco's modified Eagle's medium (DMEM, Celbio) supplemented with 10% heat

inactivated fetal calf serum (FCS), 5% glutamine and antibiotics. Cardiomyocyte cultures were prepared from 1- to 3-day old neonatal Wistar rat hearts, as described previously [9]. Before the treatments, the cells were serum starved for 18 h in medium containing 1% FCS. All treatments were dissolved in phosphate-buffered saline (PBS) or DMSO at a 1000x concentration and added to cell cultures (0.1% with respect to the total volume) in order to obtain the required concentration in the medium. Control cells received the corresponding volume of the vehicle. Cell viability was determined by trypan blue exclusion by counting living cells and stained dead cells with a Burker hemocytometer.

Western Blotting

H9c2 cells were collected in lysis buffer (5 mM dithiothreitol, 2 mM EDTA, 0.1% CHAPS, 0.1% Triton X-100, and protease inhibitors in 20 mM HEPES pH 7.5) and subjected to two cycles of freeze-thawing. Rat hearts were homogenised in 4 vols of the same buffer by an ultra Turrax device. The homogenate was then centrifuged at $15,000 \times g$ for 15 min and the supernatant, diluted in loading buffer (2% SDS, 5% glycerol, 0.002% bromophenol blue, 4% β -mercaptoethanol in 0.25 M Tris-HCl, pH 6.8), and then denatured by boiling for 4 min. Aliquots corresponding to 130 μ g protein were analyzed by SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane and probed with the specific primary antibody. After further washing, the membrane was then incubated for 1h with peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz). Immunoreactive bands were visualized by chemiluminescence with the ECL reagent (Amersham). Antibodies against phospho-Acetyl Coenzyme A Carboxylase β (ACC) (Ser79 in human), AMPK α 1/ α 2 and phospho-AMPK α 1/ α 2 (Thr172 in human) were from Cell Signaling. Anti-Akt and anti β -actin were obtained

from Santa Cruz. β -Actin was used as an internal control. Quantitative assay of immunoblotting was obtained by densitometry with a Fluor-S Max Multimager instrument (Bio-Rad).

ODC activity

To measure the activity of ODC, the cells were washed with PBS and scraped in a buffer consisting of 0.1 mM EDTA, 0.02 mM pyridoxal phosphate, 2.5 mM dithiothreitol in 10 mM sodium phosphate buffer, pH 7.2. The cells were disrupted by 3 cycles of freeze-thawing and then centrifuged at 11,000 rpm for 15 min. ODC activity in the supernatant was measured in duplicate by estimating the release of $^{14}\text{CO}_2$ from [14C-Carboxyl]-ornithine during 60 min incubation [28]. Specific ODC activity is expressed as units/mg protein, where 1 unit corresponds to 1 pmol of CO_2 /min of incubation. The amount of protein in samples was determined by Coomassie blue staining.

Cell metabolites

Acid-soluble metabolites were extracted in ice cold 0, 3M perchloric acid and measured by HPLC. Separations were achieved by a 25 cm x 4.6 mm Gemini 5 μ column from Phenomenex. Polyamines were determined in acidic cellular extracts by reversed phase HPLC after derivatization with dansyl chloride [28]. The cellular content of ATP and other nucleotides was determined after extract neutralization with buffered 3M KOH following conversion into fluorescent etheno-derivatives [29].

RNA interference

The sequences of siRNAs directed against the catalytic subunits of rat AMPK (Obtained from Sigma-Genosys) were as follows: for AMPK α 1, 5'-CUUAUUGGAUUUCCGAAGUTT-3'; for AMPK α 2, 5'-

GACAUUAUGGCGGAGGUGUTT-3'. In most experiments, a siRNA against Luciferase was used as negative control (5'-AACUUACGCUGAGUACUUCTT-3'). In some cases, the control siRNA-A, purchased from Santa Cruz Biotechnology, was used. Cells at 50 % of confluence were transfected with a final concentration of 100 nM siRNA for 24 h by Transfection reagent (Santa Cruz) according to manufacturer's instructions. 24 hours after transfection, cells were then treated with the designated drugs.

Data analysis

All the experiments shown were performed independently at least three times with comparable results. The blots are representative of results obtained in multiple experiments. All the data presented in graphs are expressed as means \pm S.E. of the mean of the indicated numbers of independent determinations. Statistical comparison between two groups were done by Student's unpaired two-tailed t test. Differences were considered as significant when $P < 0.05$.

RESULTS

Adrenergic agonists such as phenylephrine and isoproterenol cause in H9c2 cardiomyoblasts a hypertrophic response similar to that evoked in primary cardiomyocytes and in the whole heart [30-32]. First, the effect of isoproterenol on AMPK activation was studied. Analysis of soluble extracts prepared from whole H9c2 cell lysates showed increased AMPK- α (Thr172) phosphorylation following treatment with isoproterenol (10 μ g/ml). The experiment depicted in Fig. 2A shows that an increased phosphorylation of AMPK α catalytic subunits could be observed as soon as 15 min. This effect remained detectable for 2-3 h and was not associated to any changes in the level of ATP, ADP and AMP, that were unchanged (not shown). AMPK

phosphorylation was also increased (Fig. 2B) in response to phenylephrine and the physiological effector, norepinephrine (both 10 μ g/ml), as per previous reports [23,33].

Treatment with isoproterenol increased ODC activity in H9c2 cells with a timing identical to that observed in rat cardiomyocytes [7] and in the whole heart [4]. The increase in enzyme activity was very rapid and became significant between 15 and 30 min after treatment (Fig. 2A). After 4 h of treatment, putrescine, the product of ODC catalysis, was increased (Fig. 2B), whereas the major polyamines spermidine and spermine were unchanged (not shown). The increase in ODC activity was blocked by cycloheximide, an inhibitor of protein synthesis (Fig. 2C). ODC is a short-lived protein, whose level can be regulated by the rate of its proteasomal degradation [34]. In the presence of the proteasome inhibitor MG132, basal ODC activity was increased as expected, however, isoproterenol continued to increase ODC activity (Fig. 2D), indicating that change in protein stability was not involved in the response to isoproterenol. These data show that isoproterenol induced ODC activity by increasing the synthesis of new enzyme protein, accordingly to the most common mechanism of ODC regulation [34].

In order to evaluate the putative signal transduction pathways involved in ODC induction in isoproterenol-treated H9c2 cells, we tested the effect of some pharmacological inhibitors. Table 1 shows that PD98059 (50 μ M), inhibitor of the MAPK cascade, H89, a PKA inhibitor, and KN93, a CAMK-II inhibitor, were without any significant effect. On the contrary, ODC induction by isoproterenol was abolished in cells treated with the PI3K/Akt pathway inhibitor Wortmannin or with the dual PI3K/mTOR inhibitor BEZ235, suggesting a central role of Akt-mediated signal in the ODC response to isoproterenol. To determine whether the observed AMPK activation

could influence the response of ODC, H9c2 cardiomyoblasts were exposed for 2 hours to isoproterenol in the presence of AMPK inhibitors AraA [35] or STO-609. This last compound is a CAMKK β inhibitor, a proven powerful direct inhibitor of AMPK [36]. Interestingly, ODC activity resulted slightly increased in cells incubated in the presence of either AraA or STO-609. On the contrary, the AMPK activator AICAR (4 mM) and especially metformin (5 mM) slightly decreased ODC activity. Phenformin, a more potent analogue of metformin, caused a dose dependent inhibition of ODC activity. These late results suggest that AMPK activity could be associated to decreased ODC activity. On the contrary, ODC inhibition with 0,1 mM α -difluoromethylornithine (DFMO) did not induce any effect on AMPK level or phosphorylation in either control cells or isoproterenol-treated cells (not shown).

Since the specificity of pharmacological inhibitors/activators always remains limited, to support the suggestion of a modulating effect of AMPK on ODC, a siRNA approach was used. The cells were treated with two siRNAs respectively targeted against the $\alpha 1$ and $\alpha 2$ isoforms of the AMPK catalytic subunit in order to knock down AMPK enzymatic activity, or with a siRNA directed against luciferase (control siRNA). The cardiac myoblasts were then exposed for 2 hours to isoproterenol. Fig. 3A shows that a significant degree of reduction in AMPK α level was obtained in cells pretreated with both siRNAs against $\alpha 1$ and $\alpha 2$ isoforms of AMPK α , that lead to a significant reduction in cellular AMPK activity, as shown by the decrease in the phosphorylation of ACC (Ser79), a physiological AMPK substrate. The knock-down of only one isoform of AMPK α did not influence ACC (Ser79) phosphorylation.

When both $\alpha 1$ and $\alpha 2$ isoforms of AMPK were silenced, there was a marked increase in ODC activity in H9c2 cells treated with isoproterenol, along with the

decrement in AMPK activity. siRNA-mediated downregulation of a single isoform resulted to have a weaker effect on ODC activity, but the effect of the siRNA against $\alpha 2$ isoform was more pronounced when compared to the $\alpha 1$ isoform. Pretreatment with $\alpha 1$ plus $\alpha 2$ siRNAs also increased ODC activity in response to norepinephrine and phenylephrine (Fig. 3B). Fig. 3C depicts that the treatment with $\alpha 1$ plus $\alpha 2$ siRNAs did not influence the expression of ERK 1/2 or Akt, that are often associated to the growth promoting effect of adrenergic stimulation of cardiac cells [37,38].

DISCUSSION

AMPK, that is allosterically activated by AMP and phosphorylated by upstream kinases, represents the link between cell growth and energy availability [12-14]. It is generally assumed that AMPK activates mechanisms that favor the formation of ATP both directly and indirectly, and contrastingly blocks mechanism that consume ATP, such as protein synthesis [17]. Because of its homeostatic role, AMPK could essential contrast the hyperplastic as well as hypertrophic growth. The heart can sustain two types of hypertrophic growth, one termed physiological hypertrophy also known as the athletic heart, that is reversible once the cardiovascular stress ceases. The second type of hypertrophy is termed systolic pathological, it is the outcome of the body's effort to adapt to an increase in afterload usually due to an increase in blood pressure and or valvular insufficiency. This particular type of hypertrophy is not reversible, and eventually leads to cardiac dilation ,and then finally congestive heart failure [1]. Interest in defining AMPK's exact role in protein synthesis and therefore hypertrophy in cardiac cells lays in the hope of finding a key to pathological hypertrophy. To evaluate the role of AMPK in cardiac hypertrophy, we have studied the response of AMPK in a cell model of induced hypertrophy represented by H9c2 cardiomyoblasts treated

with isoproterenol.

Our findings demonstrated that treatment with adrenergic agonists, such as isoproterenol, quickly activates in cardiac cells two mechanisms that seem to contrast one another. The first one, increase in ODC activity, is linked to cell growth, whereas the second, AMPK activation, is a homeostatic mechanism that negatively modulates the first.

ODC is the enzyme responsible for the biosynthesis of polyamines, that are known to be critical in the cell cycle, showing a marked increase during all cellular growth processes [39], and have a role in the development of cardiac hypertrophy [3-8]. We have shown that isoproterenol induces ODC activity in H9c2 cells, exactly like in other cardiac models, by promoting the synthesis of the enzyme. ODC is one of the most finely regulated enzyme [34] and its synthesis is modulated by the mTOR-p70s6K pathway [40], that is activated by isoproterenol in cardiomyocytes [41]. Actually, inhibition of the PI3K/Akt pathway, that leads to mTOR activation, blocked ODC induction by isoproterenol. Inhibition of mTOR represents the main mechanism of the anti-hypertrophic effect of AMPK [12,17] and is probably also the mechanism responsible for the negative modulation of ODC.

The question is why two apparently contrasting mechanisms are activated at the same time in heart cells committed to hypertrophy. We suspect that AMPK plays a regulatory role in the transition towards the hypertrophic phenotype. The rapid activation of AMPK following adrenergic stimuli is probably due to the cells effort to guarantee itself energetic substrates in order to respond to an increase in ATP demand. The lack of optimal nutritional conditions (energetic substrates) due to a prolonged activation of AMPK could instead contrast the establishment of hypertrophy, possibly

also by means of the negative modulation of ODC activity.

We have recently provided data suggesting that adrenergic stimulation of heart cells along with the hypertrophic program also promotes self-protecting and self-regulating mechanisms [33]. The modulation of ODC activity by AMPK fits well in this model, since it could represent a powerful self-regulatory mechanism, that may represent a target for the pharmacological control of heart hypertrophy.

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TABLES AND FIGURES

Table 1. Effect of pharmacologic protein kinase inhibitor/activator on ODC activity in untreated and isoproterenol-treated H9c2 cardiomyoblasts.

<i>Treatment</i>	<i>Addition</i>	<i>Effect</i>	<i>Target</i>	<i>ODC activity (U/mg)</i>
- (ctrl)				13 ± 3
ISO				34 ± 6
-	Wortmannin (2 µM)	inhibitor	PI3K	8 ± 2
ISO	Wortmannin (2 µM)	inhibitor	PI3K	9 ± 2
-	BEZ235 (0.2 µM)	inhibitor	PI3K/mTOR	7 ± 3
ISO	BEZ235 (0.2 µM)	inhibitor	PI3K/mTOR	6 ± 2
-	PD 98059 (50 µM)	inhibitor	ERK1/2	13 ± 2
ISO	PD 98059 (50 µM)	inhibitor	ERK1/2	37 ± 4
-	H89 (5 µM)	inhibitor	PKA	15 ± 2
ISO	H89 (5 µM)	inhibitor	PKA	37 ± 4
-	KN93 (2 µM)	inhibitor	CAMKII	12 ± 3
ISO	KN93 (2 µM)	inhibitor	CAMKII	32 ± 5
-	STO 609 (1 µM)	inhibitor	CAMKKβ/AMPK	16 ± 3
ISO	STO 609 (1 µM)	inhibitor	CAMKKβ/AMPK	40 ± 4
-	AraA (0.5 mM)	inhibitor	AMPK	16 ± 2
ISO	AraA (0.5 mM)	inhibitor	AMPK	41 ± 5
-	AICAR (4 mM)	activator	AMPK	11 ± 3
ISO	AICAR (4 mM)	activator	AMPK	29 ± 4
-	Metphormin (5 mM)	activator	AMPK	9 ± 2
ISO	Metphormin (5 mM)	activator	AMPK	23 ± 4
-	Phenformin (0.5 mM)	activator	AMPK	12 ± 2
ISO	Phenformin (0.5 mM)	activator	AMPK	30 ± 4
-	Phenformin (1 mM)	activator	AMPK	9 ± 2
ISO	Phenformin (1 mM)	activator	AMPK	26 ± 3
-	Phenformin (2 mM)	activator	AMPK	8 ± 1
ISO	Phenformin (2 mM)	activator	AMPK	21 ± 3

ODC activity was determined in extracts from cells incubated 2 h in the absence or presence of 10 µM isoproterenol (ISO) and the indicated compounds. Results are means ± s.e.m. of triplicate determinations.

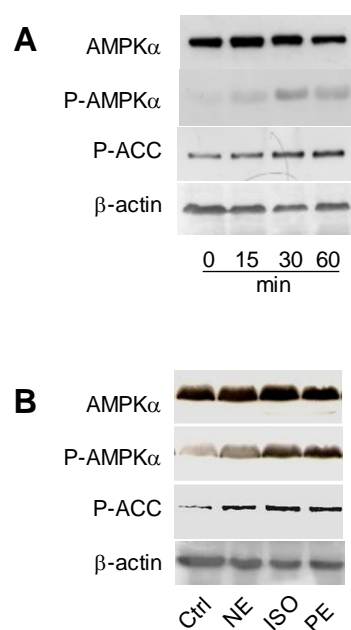


Figure 1. AMPK phosphorylation is increased by isoproterenol in H9c2 cardiomyoblasts. (A) The cells were incubated in the absence or presence of 10 μ M isoproterenol for the indicated time. Cell lysates were analysed by Western blotting with antibodies against α subunits of AMPK (AMPK α) or Thr172-phosphorylated α subunits of AMPK (P-AMPK α), or phospho-ACC (Ser79). (B) The cells were incubated for 1 h in the absence or presence of 10 μ M Norepinephrine (NE), or isoproterenol (ISO), or phenylephrine (PE), then cell extracts were analyzed by Western blotting.

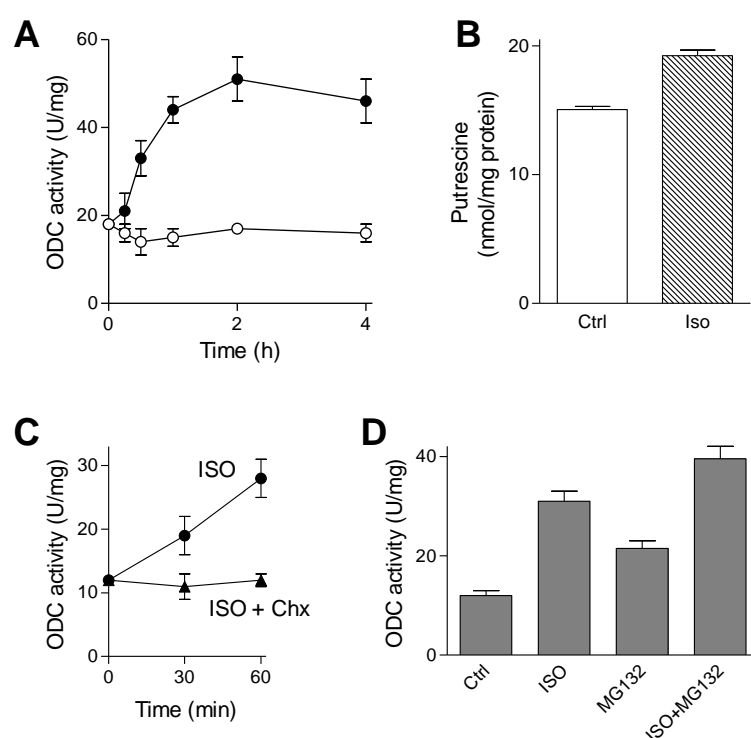


Figure 2. ODC activity is increased by isoproterenol in H9c2 cardiomyoblasts. (A) The cells were incubated in the absence (empty symbols) or presence (black symbols) of 10 μ M isoproterenol for the indicated time. Cell lysates were then assayed for ODC activity. (B) The content of putrescine was measured by HPLC in acid extracts from cells incubated for 4 h in the absence or presence of 10 μ M isoproterenol (ISO). (C) H9c2 cells were treated with 10 μ M isoproterenol in the absence or presence of 0.2 mM cycloheximide (Chx) to inhibit protein synthesis. The cells were collected at the indicated time for the assay of ODC activity. (D) Control cells and isoproterenol-treated cells (10 μ M) were incubated 2 h in the absence or presence of a 10 μ M concentration of the proteasome inhibitor MG132. The cells were then collected and ODC was assayed in cell extracts. Results are means \pm s.e.m. of three to five measurements.

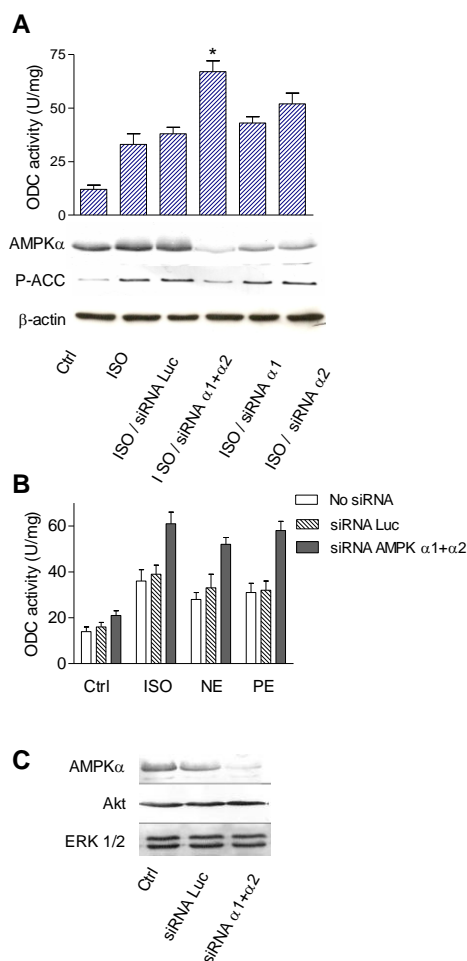


Figure 3. AMPK knock-down by siRNA causes an increase in ODC activity in isoproterenol-treated H9c2 cardiomyoblasts. (A) The cells were transfected with a control siRNA against luciferase (Luc), or AMPK $\alpha 1$ plus AMPK $\alpha 2$ siRNAs, or AMPK $\alpha 1$ siRNA, or AMPK $\alpha 2$ siRNA. The cells were then incubated in the absence or presence of 10 μ M isoproterenol. The amount of AMPK α and phospho-ACC was determined after 1 h, whereas ODC activity was measured after 2 h of isoproterenol treatment. Results of ODC activity are means \pm s.e.m. of three determinations, whereas the blots are representative of three experiments. (B) H9c2 cells transfected with the luciferase siRNA (Luc) or AMPK $\alpha 1$ plus AMPK $\alpha 2$ siRNAs, were incubated for 2 h in the absence or presence of 10 μ M Norepinephrine (NE), or isoproterenol (ISO), or phenylephrine (PE), then ODC activity was measured in cell extracts. (C) The contents of AMPK α , Akt, and ERK 1/2 were determined in cells transfected with the luciferase siRNA (Luc) or AMPK $\alpha 1$ plus AMPK $\alpha 2$ siRNAs.

CHAPTER 3: ADIPOQ SNP45 ASSOCIATED WITH LEAN BODY MASS IN PHYSICALLY ACTIVE NORMAL WEIGHT ADOLESCENT GIRLS

INTRODUCTION

Obesity research has lead to a change in viewing fat mass instead of as dead weight and fat storage, but as an endocrine organ able to secrete hormones, termed adipokines, that affect metabolism and inflammation. Adiponectin is a recently discovered adipokine that is specifically and highly expressed in human adipose tissue (Maeda et al., 1996). Adiponectin is a 247-amino acid peptide with a collagenous domain at the n-terminus and a globular domain that shares substantial homology with the subunits of complement factor C1q (Hu et al., 1996; Nakano et al., 1996; Scherer et al., 1995). What makes adiponectin a particularly interesting adipokine is that population studies show that while the majority of secreted hormones increase as fat mass increases, adiponectin, on the other hand is inversely related with fat mass, and its decrease is independently associated to the metabolic syndrome (Stenholm et al. 2010; Gnacinska et al., 2009).

Data from human studies indicate that adiponectin has insulin-enhancing as well as anti-inflammatory actions (Kadowaki and Yamauchi, 2005). Adiponectin levels are markedly reduced in obese/diabetic mice, and injection of the adiponectin globular domain to these animals ameliorates insulin resistance, an effect that can be ascribed to an enhancement of fatty acid– oxidation in skeletal muscle and a decrease of hepatic gluconeogenesis mediated by AMP-activated protein kinase (Yamauchi et al., 2001; Maeda et al., 2002; Berg et al., 2001; Combs et al., 2004). Furthermore, adiponectin

knockout mice show increased susceptibility to diet-induced insulin resistance as well as injury-induced arterial stenosis and neointimal formation. Although, the decrease in adiponectin is due to underlying disease processes, there is belief that hypoadiponectinemia is a determinant factor rather than a consequence of these conditions (Menzaghi et al., 2007).

The specific gene coding for adiponectin, officially named *ADIPOQ*, is placed on chromosome 3q27. *ADIPOQ* includes three exons, spanning a total of 16 kb of genomic sequence. Hara et al. (2002) were the first to genotype the *ADIPOQ* in search of specific loci related to obesity, adiponectin levels, and diabetes mellitus. They reported that two single nucleotide polymorphism (SNP), +45G/T and +276T/G, were related to diabetes mellitus, and that specifically the SNP 276 is also related to adiponectin serum levels as well as obesity, in the Japanese population (Hara et al., 2002). Upon this discovery, these specific loci have been investigated in many other cultural and racial populations, Korean (Hwang et al., 2009), elderly (Yang et al., 2007), Asian Indians (Vismaleswaran et al., 2008), white Caucasian and African Americans (Woo et al., 2006), Pima Indians (Vozarova de Courten et al., 2005) and obese children (Bouatia-Naji et al., 2006).

These two SNP's are reported to be statistically different from one another in obese children independent of their fat mass and have also been found to be related to insulin resistance (Bouatia-Naji et al., 2006; Verduci et al., 2009); it is imperative to define the factors that lead to childhood obesity in efforts to prevent and to also avert adult obesity. Menzaghi et al. (2007) suggested that adiponectin is a determinant factor in the development of obesity and consequently the metabolic syndrome. If this statement is true and these polymorphisms genetically predispose individuals to develop

metabolic syndrome, these two sites can be a future target for gene therapy. Therefore, the aim of this study was to determine if these two polymorphisms (+45 T/G and +276 G/T) are indeed determinants in the development of metabolic disorders or if they are secondary to other confounding factors. To do so we have selected an adolescent female population devoid of any metabolic diseases or confounding factors, such as obesity or inactivity in order to better attribute any findings to the genotype effects; a sample in which we have previously examined other variables associated with cardiovascular and metabolic health (Gruodyte et al 2010).

MATERIALS AND METHODS

Subjects

This cross-sectional study involved 170 healthy adolescent girls, aged 13-15 years, recruited from different schools and sport clubs in Estonia as described by Gruodytė et al. (2010). All girls were healthy and active participants of various types of physical activity. In order to exclude any confounding factors that might influence metabolic health we strictly recruited participants that were free from present or past diseases known to affect metabolism, and they did not use oral contraceptives, and were non smokers, two factors discovered to vary adiponectin serum levels (Luque-Ramírez et al., 2009; Kawamoto et al., 2010). Girls were also asked not to change their eating habits. Each girl and relative parent (or legal guardian) received a full written description of the nature of the study and signed an informed consent releasing the child to participate in the study. The study was approved by the Medical Ethics Committee of the University of Tartu (Estonia).

Anthropometry and sexual maturation

Body height was measured to the nearest 0.1 cm using the Martin's metal anthropometer. Body mass of the girls, wearing light clothes and no shoes, was measured using medical balance scale (A&D Instruments Ltd, UK) to the nearest 0.05 kg. Body mass index (BMI) was calculated (kg/m^2). Pubertal development of the participants was assessed by self-report using an illustrated questionnaire of pubertal stages according to the criteria of Tanner (Tanner et al., 1962) which has been previously validated (Matsudo and Matsudo, 1994) and used in previous studies (Jurimae et al., 2006). The girls were given photographs, figures and descriptions of breast and pubic hair development stages, and asked to choose the one which most accurately reflected their appearance. If a disagreement between the development of breast and pubic hair was found, the final decision was made according to the breast (Matsudo and Matsudo, 1994; Jurimae et al., 2006). The girls were also asked if they had experienced menarche.

Body Composition

Fat scan mode was used on dual-energy X-ray absorptiometry (DXA; DPX-IQ, Lunar Corporation, Madison, WI, USA). Participants were scanned in light clothing while lying flat on their backs with arms at their sides. Standard subject positioning were used for total body measurements and analyzed using the extended analysis option. DXA measurements and results were evaluated by the same examiner. Coefficients of variations for body composition parameters were less than 2%.

Blood analysis

Venous blood samples to determine the concentration of adiponectin and insulin, as well as glucose were drawn between 0730 and 0830 a.m. after an overnight fast. For those girls who had menarche, the fasting blood samples were drawn in the early

follicular phase of the menstrual cycle, i.e. days 5-7 after menstrual bleeding started (Soot et al., 2006). The whole blood and serum was separated and stored at -20 °C for later analysis. The levels of total adiponectin concentration were analyzed using ELISA kits (AdipoGen Aspen Haus, Germany). The intra- and interassay coefficients of variation (CVs) for adiponectin were less than 5%. The concentrations of insulin were analyzed on Immulite 2000 (DPC, Los Angeles, CA, USA). The intra- and interassay CVs were less than 5% and 12%, respectively, at an insulin concentration of 6.6 μ IU/ml. Glucose concentration was measured using the Hexokinase/Glucose-6-Phosphate Dehydrogenase method with a commercial kit (Boehringer, Mannheim, Germany). In addition, the insulin resistance index was calculated using homeostasis model assessment (HOMA): fasting plasma insulin (μ IU/ml) x fasting plasma glucose (mmol/l)/22.5 (Matthews et al., 1985). The greater HOMA values indicate the greater level of insulin resistance.

Genotyping

Genomic DNA for analyses was extracted from 1ml of the subjects' whole blood using QIAamp DNA Blood Midi Kit (Qiagen, Germany). DNA was prepared for amplification adding forward and backward primers with Master Mix (Solis Biodyne, Estonia). SNP +45 (T/G) in the ADIPOQ gene was genotyped by the amplification of genomic DNA using the following primers: forward, 5'-GCA GCT CCT AGA AGT AGA CTC TG-3' and reverse, 5'-TCT GTG ATG AAA GAG GCC AG-3' (DNA Technology A/S, Denmark). The PCR cycling was performed in a Biometra T Gradient Thermoblock (Biometra, Germany) as follows: 94 °C for 10 min, 42 cycles of 30s at 94 °C, 30s at 57 °C, 30 s at 72 °C, 10 min extension step at 72 °C, lid temperature 98 °C. The PCR fragment was digested with the enzyme SMAI (Fermentas, Vilnius, Lithuania) at

30 C for 16 h. SNP +276 (G/T) in the ADIPOQ gene was genotyped by the amplification of genomic DNA using the following pair of primers: forward, 5'-TCT CTC CAT GGC TGA CAG TG-3' and reverse, 5'-AGA TGC AGC AAA GCC AAA GT-3' (DNA Technology A/S, Denmark). The PCR cycling was performed as follows: 94 C for 10 min, 35 cycles of 30s at 94 C, 30 s at 55 C, 30s at 72 C, and 10 min extension step at 72 C. The polymorphism was typed using the enzyme BsmI (Fermentas, Vilnius, Lithuania) at 37 C for 16 h. Restricted products were analyzed by electrophoresis in 1.5% agarose gel. The agarose gel was then stained with ethidium bromide and exposed under UV light (Uvitec, England) in order to visualize the digested fragments.

Statistical analyses

The independent segregation of alleles was tested for the Hardy-Weinberg equilibrium with a χ^2 test, and both genotypes were considered consistent. Normality of adiponectin, insulin, fat, and lean body mass were controlled by one sample Kolmogorov-Smirnov test. If analyses were $p > 0.05$ further analyses were parametric. If on the other hand the $p < 0.05$ in the KS test, analyses were treated with non parametric measures. To determine differences between genotype group for normally distributed BMI, fat mass, lean body mass, adiponectin, and glucose levels, a one way analysis of variance (ANOVA) was performed, and for the non normally distributed values, insulin and HOMA index, Mann-Whitney U test was applied. Tests were not adjusted and adjusted for fat mass, age. All statistical analyses were performed with SPSS statistical package, version 10.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

General characteristics of the subjects

General characteristics of the 170 adolescent female subjects are presented in Table 1. Mean values for fat mass, lean body mass, fasting glucose, and adiponectin serum levels were all within reference ranges and were normally distributed as per Kolmogorov-Smirnov test. Insulin and HOMA values were significantly different from Kolmogorov-Smirnov test and therefore not considered as normally distributed data.

Frequency of the 45T/G and 276G/T ADIPOQ polymorphisms

Frequency of each polymorphism in the population is reported in Table 1. These genotype distributions were in Hardy-Weinberg equilibrium ($P = .598$ at 45T/G, $P = 0.102$ at 276G/T).

Pearson and Spearman rho correlation of HOMA and serum adiponectin with anthropometric parameters and metabolic variables

We found that in this rather lean population there was no significant correlation between adiponectin and fat mass and BMI as expected, but only a tendency: fat mass ($r = -0.154$, $p = 0.056$), BMI ($r = -0.147$, $p = 0.059$). Also reported is a negative correlation between HOMA and age ($r = -0.177$, $p = 0.032$) (Table 2).

Metabolic and anthropometric parameters according to Tanner Stage

Metabolic and anthropometric parameters according to genotype

There were no genotype-related differences in Tanner stage, BMI, fat mass, adiponectin, glucose, insulin, and HOMA values at positions 45 and 276. However compared with homozygous TT carriers, TG carriers on position 45 had lower lean body mass (TT 41.1 ± 5.2 ; TG 36.9 ± 5.4 , $P = 0.012$) (Table 3).

DISCUSSION AND CONCLUSIONS

The main finding of the current study was that there were no Tanner stage, BMI, fat mass nor biochemical parameters differences among genotype groups in our normal

weight population as reported in other studies; however, G allele carriers of the 45 G/T genotype had significantly lower lean body mass compared to TT genotype (36.9 ± 5.4 kg and 41.1 ± 5.2 kg, respectively; $p < 0.05$).

To control for any possible effect of puberty on other metabolic factors Tanner stage mean was determined for each genotype. Studies shows there is an increase in fat mass as Tanner stage increases from 1-5 (Vink et al., 2009); there were no differences in Tanner stage among genotype groups in our population ($p > 0.05$), allowing us to exclude any pubertal effect on any significant changes found among genotype.

In the preceding articles concerning subjects afflicted by one or more than one component of the metabolic syndrome regardless of age or ethnicity, the 45 T/G was associated with adiponectin levels and 276 G/T was related to insulin resistance. Specifically, the G allele of SNP 276 G/T was found to be associated with low plasma adiponectin levels and higher insulin resistance index in obese Japanese individuals as well as in obese children (Hara et al., 2002; Bouatia-Naji et al., 2006). Additionally, subjects with the G/T or G/G genotype at position 45 were reported to have a significantly higher risk for type 2 diabetes in the obese Japanese population (Hara et al., 2002.). However, just as in our study, none of the associations between polymorphism and various risk factors were present in the normal weight subjects (Hara et al., 2002; Jang et al., 2005). Furthermore, in the meta-analysis conducted by Menzaghi et al. (2007), in which the sample size was over 2,000 individuals, no relationships between the aforementioned genotypes and metabolic risk factors were found in the subjects with normal BMI. Since our results confirm Menzaghi et al. (2007), findings regarding normal weight individuals. Although the power of our study was rather low (average 0.45), therefore increasing the possibility that the null

hypothesis, no difference among groups, is false, our findings or lack of significance difference of any metabolic parameters among genotype groups is in line with research published considering our population only consisted of lean subjects. We therefore stand by our results considering that all previous published studies with a stronger power support these findings. We can then conclude that the ADIPOQ polymorphisms 276 G/T and 45 T/G do not seem to be determinants of the metabolic syndrome in normal weighted healthy subjects, and that the effects of these polymorphisms observed in obese populations are secondary to other confounding factors, such as diet and physical activity.

Although, there was no significant relationship between the metabolic risk parameters (BMI, HOMA, adiponectin levels) and the polymorphisms analyzed in our sample of normal weight active adolescent females, there was a significant difference in lean body mass between genotypes, expressly the SNP +45 G/T. Subjects who had the G allele substitution on site 45 had significantly lower lean body mass when compared with subjects with the homogenous TT wild type genotype (TG:36.90/TT:41.07 kg, $P=0.012$). Similar findings are not found in the literature, because to date all previous published studies have not reported lean body mass, but instead BMI or other anthropometric measures of metabolic health (waist circumference, percent body fat) when investigating these two genotypes. BMI, however, is not suitable to be used to predict fat mass in non obese subjects (Freedman et al., 2009) because increase in BMI can be caused by increase in lean body mass. Although the other studies main focus was differences between the obese population and respective controls, it would have been more precise, to have calculated fat mass and lean body mass independently, especially

since adiponectin is produced by adipocytes and because they did have normal weight individuals as part of their cohort.

The significance of our novel relationship found, G allele at site 45 was associated to a decrease in lean body mass, lies in the fact that previous studies have showed that the G allele (both G/T and GG genotype) at site 45 was associated with an increase risk to Type II diabetes (Hara et al., 2002). Not only have cross sectional studies seen this increase in risk for diabetes in G allele carriers on site 45 trend, but as well as prospective studies (Fumeron et al., 2004; Tso et Al., 2006; Zacharova et al., 2005). Menzaghi et Al (2007) did not however confirm this association, and explained this difference between findings on the environmental and genetic factors seen in the various population in the cross sectional studies; Menzaghi et Al (2007) was unable, however, to explain the fact that the prospective population studies did indeed find the aforementioned correlation, while their statistical analysis did not. This question mark regarding this specific association leaves open the possibility that this association does indeed exist, but possibly through an indirect pathway, not yet discovered.

Our findings offer a possible hypothesis as to the fact the the G allele on site 45 could lead to the increased risk of Type II diabetes through a decrease in lean body mass. It has been shown that increase in muscle mass leads to an improved glucose metabolism (List et al., 2009) and that resistance training is more effective in treating insulin resistance and type 2 diabetes mellitus than aerobic exercise (Winnick et al., 2008). Further, it has been found that electro-stimulated muscle movement in spinal cord patients improved their insulin resistance since the cause of the type 2 diabetes mellitus in the spinal cord in injured population is due to the extensive amount of atrophy in their muscles (Winnick et al., 2008). Physiologically, an increase in muscle

mass is associated with a high concentration of GLUT-1 and GLUT-4 which are also associated with higher insulin sensitivity and glucose metabolism (Jeon et al., 2002).

Aside from the muscular physiology demonstrating the key role of lean body mass in the prevention of Type II diabetes, there are also biochemical studies that demonstrate a direct role of adiponectin and muscle metabolism. Iwabu et al (2010) reported that adiponectin caused an increased activity in the SIRT-1, AMP-activated kinase (AMPK) phosphorylation, peroxisome proliferator-activated receptor- γ coactivator (PGC)-1 α deacetylation, mitochondrial biogenesis, increase oxidative metabolism, increase insulin sensitivity. Such pathway was inactive when adiponectin stimulus was given to muscle adiponectin receptor knock-out mice leading to insulin resistance (Iwabu et al., 2010). Evidence that correct muscle metabolism is imperative to insulin sensitivity (List et al., 2009, Winnick et al., 2008, Jeon et al., 2002) and that adiponectin has an potent role in the muscle metabolism by increasing mitochondrial activity and biogenesis through the deacetylation of PGC-1 α (Iwabu et al., 2010) and the well documented translocation of glucose transporter type (GLUT)-4 and expression to the cell surface via AMPK phosphorylation (Iwabu et al., 2010; Parks et al., 2009; Li J et al., 2004). Our findings therefore suggest that the plausible hypothesis for the increased risk in type 2 diabetes mellitus seen in the population that carry the G allele at site 45 of ADIPOQ can be due to a decrease in lean body mass, sign of decrease muscle metabolism, and not to the genotype itself.

A valid limitation of this study that should be addressed is the size of our cohort. The natural frequency of the ADIPOQ polymorphism in Caucasians is (Lilioja et al., 1987) resulted in a small number of participants genotyped with the homozygous allele substitution: 11 subjects found with the TT genotype at site 276 and no subjects were

found to have the homozygous GG substitution at site 45 (natural frequency of GG genotype is 3.2%); however, results reported were shown to be statistically significant. A presence of a sufficient amount of subjects that contained the homozygous G substitution genotype could have revealed other valid relationships or even strengthened the relationship found between the G allele and lean body mass and not just the GT genotype. For this reason, it would be necessary to confirm our findings in a sufficiently large sample size that guarantees a sufficiently statistically large amount of subjects that have the homozygous G substitution on site 45. Also, although to date no studies have been conducted, possibly due to the difficulty of obtaining this type of invasive data on a population large enough to conduct a genotype study, it would be of even greater importance to evaluate biochemical parameters, markers of mitochondria metabolism among various genotype groups.

In conclusion, the relationship between lean body mass, risk of insulin resistance, and SNP 45 T>G should be further investigated. The confirmation of our theory that the increase in risk of diabetes seen in the obese population G allele substitution at site 45 is due to decreased muscle metabolism, expressed by the decrease in lean body mass, present in carriers of this genotype could lead to the distinguishing of individuals who have the propensity of having low lean body mass to thereafter prescribe resistance training from an early age to therefore prevent type 2 diabetes in adulthood.

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TABLES

Table 1 Summary of parameters in peripubertal and pubertal adolescent girls (n=170)

	Mean \pm SD	Frequencies (%)
<i>Phenotype measures</i>		
Age (yrs)	14.03 \pm 1.07	
Height (cm)	164.64 \pm 7.38	
Weight (kg)	54.30 \pm 8.77	
BMI (kg/m ²)	20.00 \pm 2.50	
Fat mass (kg)	13.23 \pm 5.23	
Lean body mass (kg)	40.70 \pm 5.31	
Adiponectin (μ g/ml)	14.76 \pm 6.59	
Insulin (μ IU/ml)	6.32 \pm 4.01	
Glucose (mmol/l)	4.86 \pm 0.39	
HOMA	1.38 \pm 0.91	
Tanner stage (1/2/3/4/5)		1.2/5.4/15.5/64.9/13.1
<i>Genotype ADIPOQ</i>		
SNP 276 G>T		
% GG/GT/TT		46.3/47.9/5.9
SNP 45 T>G		
% TT/TG/GG		92.6/7.4/0

Table 2 Pearson and Spearman rho bivariate correlation of Adiponectin and HOMA values with metabolic parameters

	Adiponectin		HOMA	
	r	p	r	p
BMI	-0.147	0.059	0.13	0.116
Fat mass	-0.154	0.056	0.017	0.847
Lean body mass	-0.004	0.958	0.003	0.97
Insulin	0.071	0.274	0.988	<0.001
Glucose	0.09	0.25	0.426	<0.001
Age			-0.177,	0.032

Table 3 Means(\pm SD) of phenotype measurements grouped by genotype

	Tanner stage	Fat mass (kg)	Lean Body Mass (kg)	Adiponectin (μ g/ml)	Glucose (mmol/l)	Insulin (μ IU/ml)	HOMA
SNP 276 G>T							
GG	3.83 \pm 0.09	13.31 \pm 5.19	41.19 \pm 5.63	14.69 \pm 5.99	4.85 \pm 0.40	6.78 \pm 4.41	1.48 \pm 1.00
GT	3.82 \pm 0.09	12.88 \pm 5.50	40.36 \pm 5.05	15.70 \pm 7.52	4.88 \pm 0.40	6.15 \pm 3.54	1.35 \pm 0.80
TT	4.00 \pm 0.26	14.06 \pm 2.60	40.52 \pm 5.52	12.16 \pm 5.26	4.92 \pm 0.28	6.38 \pm 4.73	1.14 \pm 1.06
SNP 45 T>G							
TT	3.86 \pm 0.72	13.30 \pm 5.35	41.07 \pm 5.23	14.91 \pm 6.65	4.87 \pm 0.40	6.31 \pm 3.9	1.39 \pm 0.90
TG	3.45 \pm 1.29	11.16 \pm 2.96	36.90 \pm 5.35 *	16.83 \pm 7.91	4.88 \pm 0.39	7.41 \pm 5.55	1.55 \pm 1.15

* Significantly different from TT genotype p<0.05

CHAPTER 4: ASSOCIATION OF COMMON VARIANTS IN THE ADIPONECTIN GENE WITH THE RELATIONSHIPS BETWEEN SERUM ADIPONECTIN, ESTRADIOL LEVELS AND BONE DENSITY IN PHYSICALLY ACTIVE GIRLS

INTRODUCTION

Females have any interesting relationship with estradiol and adiponectin. Although different, one a sex hormone and the other an adipocytokine that is specifically and highly expressed in human adipose tissue (1), both, upon puberty, are present at a higher concentration in females when compared to males (2). Females have a 15% higher adiponectin serum level when compared to their male counterpart (3) and both seem to play a protective role in preventing against osteoporosis and cardiovascular disease (4-8).

Osteoporosis is a disease in which bone mineral density (BMD) is reduced, bone microarchitecture is disrupted, and the amount and variety of proteins in bone is altered. Bone is in constant dynamic process of formation and resorption. The imbalance that occurs after menopause leads to osteoporosis (9). Osteoporosis may be caused not only by bone mineral loss in adulthood, but also by failing to accumulate an optimal bone mass during childhood and adolescence (10). It is known that regular high-impact weight-bearing physical activity (such as sport games, dance, gymnastics, running, or jumping exercises) during puberty has beneficial effect on growing bone by increasing its formation (11, 12).

Both estradiol and adiponectin biochemically have resulted to play an important role in bone health. Estradiol diminishes oxidative stress in bone and bone marrow, attenuates the generation of osteoblasts, and decreases the prevalence of mature

osteoblasts apoptosis (13, 14). Adiponectin receptors (AdipoR1 and AdipoR2) have been reported to be expressed in human osteoblasts (15, 16) and osteoblast-like cells (17). Adiponectin mRNA expression and adiponectin secretion increases during the differentiation of human osteoblasts in culture (16). Although the biological findings suggest an adiponectin positive effect on bone health, population studies that have investigated serum adiponectin levels and bone mineral density have surprisingly reported a negative and independent relationship between adiponectin and bone mineral density, in post menopausal women (18), anorexic girls (19), and middle aged men (20). However, these previous studies fail to investigate the plausible interaction between estradiol and the negative relationship between adiponectin and bone density, and failed to explain why the relationship found in cohort studies contradict biological findings (21).

To date, two studies have investigated the gene effect of the adiponectin gene (ADIPOQ) (location 3q27.3) on bone health, however reporting opposing results. Lee et al. reported the G allele in position 45 favored a significant decrease in BMD in an adult Korean Female population (mean age 51.4 yrs), independent of age, while they found no halotype effect (22). On the contrary, Zhang et al. (2007) population study reported no gene effect of either SNP 45 or 276 on BMD, but instead found a strong gene effect on the T-T/T-T halotype on postmenopausal lumbar spine BMD (23).

Both studies regarding this topic concentrated on a population at which bone formation is either stable or decreasing (22,23). It is of our interest to understand the relationship between adiponectin and estradiol and bone health by means of optimal accrual, and to determine if optimal accrual is effected by ADIPOQ polymorphisms, as reported by Lee et al. (2006) or Zhang et al. (2007). With weight bearing exercise being

such an important component to bone formation we have specifically chosen a cohort of physically active adolescent females in whom we have previously examined other variables associated with bone metabolism such as bone mineral density and content, lean and fat mass, puberty stages, estradiol plasma, and adiponectin serum levels (24) to therefore truly understand the magnitude of any genetic effect revealed by this study.

MATERIALS AND METHODS

Subjects

This cross-sectional study involved 170 healthy adolescent girls, aged 13-15 years, recruited from different schools and sport clubs in Estonia as described by Gruodytė et al. (24). All girls were relatively healthy and active, participants of various types of physical activity (swimming, cross country skiing, rhythmic gymnastics, team sports, running). All participants were free from present or past diseases known to affect skeletal metabolism, none of the girls were receiving medications known to affect bone, and they did not use oral contraceptives. Girls were also asked not to change their eating habits. Each girl and her parent (or legal guardian) received a full written description of the nature of the study and signed an informed consent for the participation in the study. The study was approved by the Medical Ethics Committee of the University of Tartu (Estonia).

Anthropometry and sexual maturation

Body height was measured to the nearest 0.1 cm using the Martin's metal anthropometer. Body mass of the girls, wearing light clothes and no shoes, was measured using medical balance scale (A&D Instruments Ltd, UK) to the nearest 0.05 kg. Body mass index (BMI) was calculated (kg/m^2). Pubertal development of the participants was assessed by self-report using an illustrated questionnaire of pubertal

stages according to the criteria of Tanner (25), which has been previously validated (26) and used in previous studies (27). The girls were given photographs, figures and descriptions of breast and pubic hair development stages, and asked to choose the one which most accurately reflected their appearance. If a disagreement between the development of breast and pubic hair was found, the final decision was made according to the breast (26, 27). The girls were also asked if they had experienced menarche.

Bone mineral density

Bone mineral density (BMD) (g/cm^2) and bone mineral content (BMC) (g) at femoral neck and lumbar spine (L2-L4) were measured using dual-energy X-ray absorptiometry (DXA; DPX-IQ, Lunar Corporation, Madison, WI, USA) equipped with proprietary software, version 3.6. Participants were scanned in light clothing while lying flat on their backs with arms at their sides. The fast scan mode and standard subject positioning were used for total body measurements and analyzed using the extended analysis option. DXA measurements and results were evaluated by the same examiner. Coefficients of variations for the femoral neck and lumbar spine BMD and BMC measurements were less than 2%.

Blood analysis

Venous blood samples to determine the concentration of selected hormones, adiponectin and estradiol were drawn between 0730 and 0830 a.m. after an overnight fast. For those girls who had menarche, the fasting blood samples were drawn in the early follicular phase of the menstrual cycle, i.e. days 5-7 after menstrual bleeding started (28). The whole blood and serum was separated and stored at $-20\text{ }^{\circ}\text{C}$ for later analysis. The levels of adiponectin concentration were analyzed using ELISA kits (AdipoGen Aspen Haus, Germany). The intra- and interassay coefficients of variation (CVs) for adiponectin –

less than 5% and 6%, respectively. The concentrations of estradiol were analyzed on Immulite 2000 (DPC, Los Angeles, CA, USA). The intra- and inter-assay coefficients of variation were less than 7%.

Genotyping

Genomic DNA for analyses was extracted from 1ml of the subjects' whole blood (blood withdrawal described above) using QIAamp DNA Blood Midi Kit (Qiagen, Germany). DNA was successfully extracted from all samples with a concentration ranging from 10-50 ng/μl. DNA was prepared for amplification adding forward and backward primers with Master Mix (Solis Biodyne, Estonia). SNP +45 (T/G) in the ADIPOQ gene was genotyped by the amplification of genomic DNA using the following primers: forward, 5'-GCA GCT CCT AGA AGT AGA CTC TG-3' and reverse, 5'-TCT GTG ATG AAA GAG GCC AG-3' (DNA Technology A/S, Denmark). The PCR cycling was performed in a Biometra T Gradient Thermoblock (Biometra, Germany) as follows: 94 C for 10 min, followed by 42 cycles of 30s at 94 C, 30s at 57 C and 30 s at 72 C, and ending with a single 10 min extension step at 72 C, with the lid temperature at 98 C. The PCR fragment was 367 bp in length and was digested with the enzyme SMAI (Fermentas, Vilnius, Lithuania) at 30 C for 16 h. Restriction products were analyzed by electrophoresis in 3 % agarose, and digestion produced allele G: 318+148bp and allele T: 466 bp. SNP +276 (G/T) in the ADIPOQ gene was genotyped by the amplification of genomic DNA using the following pair of primers: forward, 5'-TCT CTC CAT GGC TGA CAG TG-3' and reverse, 5'-AGA TGC AGC AAA GCC AAA GT-3' (DNA Technology A/S, Denmark). The PCR cycling was performed in a Biometra T Gradient Thermoblock (Biometra, Germany) as follows: 94 C for 10 min, followed by 35 cycles of 30s at 94 C, 30 s at 55 C, and 30s at 72 C, and ending with a single 10 min extension

step at 72 C. The polymorphism was typed using the enzyme BsmI (Fermentas, Vilnius, Lithuania) at 37 C for 16 h. Restricted products were analyzed by electrophoresis 1.5% agarose gel.

Statistical analyses

The independent segregation of alleles was tested for the Hardy-Weinberg equilibrium using χ^2 test, and both genotypes were considered consistent. Normality of adiponectin, insulin, estradiol, fat, and lean body mass were controlled by one sample Kolmogorov-Smirnov (KS) test. If analyses were $p > 0.05$ further analyses were parametric. If on the other hand the $p < 0.05$ in the KS test, analyses were treated with non parametric measures. To determine differences between genotype group for normally distributed adiponectin, bone content and density parameters, fat mass, and lean mass, ANOVA was performed. Mann-Whitney U and Kruskal-Wallis test was performed to determine if there were significant differences in estradiol serum level between genotype groups. Correlations of parametric parameters were done with Pearson's partial correlation and controlled for fat and lean mass, while non parametric correlations were tested with Spearman rho. All statistical analyses were performed with SPSS statistical package, version 10.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

General characteristics of study subjects

General characteristics of the 170 adolescent female subjects are presented in Table 1. Mean values for fat mass, lean body mass, adiponectin serum levels, and all bone mineral content and density were all within reference ranges and normally distributed as per Kolmogorov-Smirnov test. Estradiol values were significantly different from Kolmogorov-Smirnov test and therefore not considered normally distributed data.

Frequency of the 45T/G and 276G/T ADIPOQ polymorphisms

Frequency of each polymorphism in the population is reported in Table 1. These genotype distributions were in Hardy-Weinberg equilibrium ($P = 0.598$ at 45T/G, $P = 0.102$ at 276G/T).

Metabolic, Tanner stage, and anthropometric parameters according to genotype

There were no genotype-related differences for Tanner stage, BMI, fat mass, percent body fat, adiponectin, and estradiol at position 45 and 276. However compared with homozygous TT carriers, TG carriers at position 45 had lower lumbar spine BMC (TT 43.36 ± 9.54 ; TG 35.45 ± 6.54 , $P=0.008$). Data presented in Table 2.

Pearson's and Spearman's correlation between bone mineral content and density measurements, hormone levels and anthropometric parameters

Correlation coefficients reported in Table 3 reveal that all anthropometric measurements are significantly correlated with all bone mineral content and density measurements. Lean body mass had the highest correlation coefficient to bone parameters than any other measurement. Adiponectin serum level was not significantly associated to any of the measurements presented, while estradiol was significantly negatively correlated with BMI ($R=-0.27$, $P=0.001$) and fat mass ($R=-0.30$, $P=0.001$) when tested for Spearman rho's correlation.

Pearson and Spearman rho correlation of serum estradiol and adiponectin with bone content and density parameters according to genotype

Subjects were grouped by genotype and then correlation coefficients were calculated mass between estradiol and adiponectin while controlling for bone mineral content and density and lean body mass and fat. There were no significant correlations between both estradiol and adiponectin, and bone health parameters when considering the whole sample, but when subdivided by genotype, significant relationships were revealed.

There was a significant correlation present between estradiol plasma levels and BMD total values when subjects were homozygous wild-type at position 276 when controlled for lean body mass and fat mass (Estradiol x BMD total $R=0.26$ $P=0.049$).

There were also significant correlations between estradiol and bone parameters when subdivided by genotype at position 45. Estradiol and lumbar BMC ($R=0.76$, $P=0.041$), and femoral neck BMC ($R=0.86$, $P=0.013$), and femoral neck BMD ($R=0.93$, $P=0.003$), and total BMD ($R=0.75$, $P=0.05$) were found significant when only subjects that were heterozygous TG were considered.

There was also an increase in significant correlations between adiponectin and bone parameters when subdivided by genotype. When controlled for fat mass and lean body mass there were significant correlations between adiponectin serum levels and lumbar BMC ($R=0.336$, $P=0.004$), femoral neck BMC ($R=0.25$, $P=0.036$), total BMC ($R=0.28$, $P=0.019$), and lumbar BMD ($R=0.25$, $P=0.037$) in the homozygous wild-type GG group at position 276. When the polymorphism at position 45 was subdivided there were significant correlations present between adiponectin and lumbar BMC ($R=0.20$, $P=0.021$) and femoral neck BMC ($R=0.17$, $P=0.042$) when subjects were homozygous wild-type TT. Data displayed in Table 4.

DISCUSSION

Our results suggest that there is an ADIPOQ gene effect in relation to bone parameters. Statistical analysis show that the presence of the T allele in position 45 favors an increase in lumbar spine bone mineral content (BMC) when compared to subjects with a G allele substitution (TT: 43.36 ± 9.54 , TG: 35.45 ± 6.54 , $P<0.05$). Both lean body mass and fat mass were controlled for when investigating the gene effect on the relationship between adiponectin and estradiol. It was found that the G allele in site

45 and 276 favored the presence of a significant positive correlation between estradiol and bone mineral density (45: $R = 0.75$; 276: $R = 0.26$, $P < 0.05$). The wild-type homozygous genotype in position 45 and 276 strengthens the positive correlation between adiponectin and various bone mineral content parameters and lumbar spine bone mineral density (45: $R = 0.21-0.22$; 276: $R = 0.25-0.34$; $P < 0.05$).

Our genotyping investigation supports the findings published by Lee et al. (2006), suggesting a novel relationship between genotype and bone parameters. More specifically the importance of the T allele at site 45 in predisposing subjects to have higher bone mineral content and density (22). We however did not find any halotype effect (data not shown) on bone mineral density, contrary to Zhang et al. (2007) findings (23).

Estrogens are stated as being the biggest contributor to bone mineral acquisition during puberty in both males and females (29), and as expected there is a strong positive correlation found between estradiol levels, Tanner stage, and bone mineral parameters (data not presented); however, since there was no difference in total estradiol levels or tanner stage among gene group, it is possible to exclude the effect of estradiol and pubertal stage on the gene effect observed on lumbar spine BMC.

We report a positive correlation present between BMI and body weight with bone mineral density and content, a correlation repeatedly supported in literature; however, we have also seen that from all anthropometric measures lean body mass is the best predictor of bone mineral density, findings supported by a cross sectional study in Sri Lanka (30). Reid et al. (1995) suggested that exercise is the cause for the dissociation between fat mass and bone mineral density (31), a characteristic found in our sample. Moreover, interestingly we also previously reported that T allele favored a

greater lean body mass (32), the same relationship found between polymorphism 45 and lumbar spine BMC with also lean body mass. The increase in lean body mass in this genotype group could very well explain the relationship found between genotype and bone mineral density in our physically active population (30,31). In addition, the importance of lean body mass is further supported by the fact, that although estradiol levels is considered to be the biggest contributor to bone formation in males and females (29) males still have a higher bone mineral density than females upon puberty even though both estradiol and adiponectin levels are higher in females than in males (3, 29, 33). A possible explanation could be the increase in lean mass in the male population that usually accompanies puberty (34).

This is the first study to date to report an ADIPOQ genetic effect on adiponectin's and estradiol's relative effect on bone mineral parameters. It would seem only obvious the existence of this relationship considering there is an increase in osteoporosis after the fall in estradiol and adiponectin levels present after menopause. Because this aforementioned relationship is also present between the increase in cardiovascular disease upon menopause, Yoshihara et al. (2009) were the first to investigate a possible genetic interaction between adiponectin and estradiol and risk for cardiovascular disease (35). Contrary to our present study, Yoshihara et al. (2009) studied the genetic effect of the estrogen receptor (ER) polymorphism and found that ER polymorphism was related to adiponectin serum levels (35). The results of this study along with those of Yoshihara et al. (2009) demonstrate the presence of a genetic interaction, although unclear, that exists between estradiol and adiponectin and their effects on bone and cardiovascular health (35). Since both hormones have anti-inflammatory effects and both increase during osteoblast differentiation (7,13, 16),

findings suggest that the effect that either of these hormones have on bone and cardiovascular are not independent from one another and therefore, in future studies of bone and cardiovascular health, both hormones should be evaluated.

Moreover, these findings also highlight the importance of lean body mass, and therefore exercise, which is why it would be opportune to follow this study with an experiment evaluating the effect of an exercise treatment on bone formation and the genotype effect of adiponectin, estrogen, and estrogen receptor polymorphisms, to help improve exercise prescription.

A valid limitation of this study is the size of our cohort, visible in the fact that in total there were only 11 subjects found with the TT genotype at site 276 and no subjects were found to have the homozygous GG substitution at site 45. The absence of a genotype group restricted us from determining if the effect seen at site 45 is linked to the G allele or is it a genotype effect seen solely in the GT genotype group. Although the power of our study was rather low (average 0.45), therefore increasing the possibility that the null hypothesis, no difference among groups, is false, our findings, or lack of significant difference of any metabolic parameters among genotype groups, is in line with research published. We therefore stand by our results considering that all previous published studies with a stronger power support these findings. It would be forthcoming to reconfirm the G allele in position 45 effects does indeed favor a decrease bone mineral density and does increase the correlation between estradiol and bone mineral density.

In conclusion the ADIPOQ single nucleotide polymorphism 45 seems to have a direct effect of lumbar spine bone mineral content and an indirect effect on how both estradiol and adiponectin levels are related to bone mineral content and density.

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TABLES

Table 1 Summary of parameters in subjects (n=170)

	Mean \pm SD	Frequencies (%)
Age (yrs)	14.03 \pm 1.07	
Height (cm)	164.64 \pm 7.38	
Weight (kg)	54.30 \pm 8.77	
Fat mass (kg)	13.23 \pm 5.23	
Lean body mass (kg)	40.70 \pm 5.31	
BMI (kg/m ²)	19.98 \pm 2.50	
BMC lumbar spine (g)	42.74 \pm 9.52	
BMC femoral neck (g)	4.81 \pm 0.77	
BMD lumbar spine (g/cm ²)	1.09 \pm 0.13	
BMD femoral neck (g/cm ²)	1.04 \pm 0.13	
Tanner stage (1/2/3/4/5)		1.2/5.4/15.5/64.9/13.1
<i>Hormonal Measures</i>		
Adiponectin (μ g/ml)	14.76 \pm 6.59	
Estradiol (pmol/l)	99.02 \pm 104.68	
<i>Genotype ADIPOQ</i>		
SNP 276 G>T		
% GG/GT/TT		46.3/47.9/5.9
SNP 45 T>G		
% TT/TG/GG		92.6/7.4/0

BMI: body mass index

BMD: bone mineral density

BMC: bone mineral content

Table 2 Means (\pm SD) and Ranks of phenotype measurements grouped by genotype

	BMC l2-l4 (g)	BMC femo (g)	BMD l2-l4 (g/cm ²)	BMD femo (g/cm ²)	Adiponectin (μ g/ml)	Estradiol (pmol/l)
SNP 276 G>T						
GG	43.64 \pm 9.59	4.88 \pm 0.76	1.11 \pm 0.14	1.05 \pm 0.11	14.69 \pm 5.99	122.21 \pm 143.36
GT	41.91 \pm 9.51	4.75 \pm 0.80	1.08 \pm 0.13	1.03 \pm 0.13	15.70 \pm 7.52	82.71 \pm 55.96
TT	43.31 \pm 10.37	4.8 \pm 0.62	1.08 \pm 0.14	1.01 \pm 0.08	12.16 \pm 5.26	77.78 \pm 59.61
SNP 45 T>G						
TT	43.36 \pm 9.54 *	4.84 \pm 0.78	1.10 \pm 0.13	1.04 \pm 0.12	14.91 \pm 6.65	99.85 \pm 108.58
TG	35.45 \pm 6.54	4.44 \pm 0.56	1.03 \pm 1.03	1.0 \pm 0.12	16.83 \pm 7.91	104.22 \pm 65.68

*P<0.05

BMC l2-l4: lumbar bone mineral content

BMC femo: femoral neck bone mineral content

BMD l2-l4: lumbar bone mineral density

BMD femo: femoral neck bone mineral density

Table 3 Pearson's and Spearman rho's correlation between bone parameters and hormone levels, and anthropometric parameters

	BMCL2- L4 (g)	BMC femo (g)	BMC tot (g)	BMD L2- L4 (g/cm ²)	BMD femo (g/cm ²)	BMDtot (g/cm ²)	Adiponectin (µg/ml)	Estradiol (pmol/l)
BMI	0.35**	0.49**	0.53**	0.41**	0.35**	0.48**	-0.15	-0.27**
Lean body mass	0.78**	0.76**	0.85**	0.64**	0.66**	0.73**	0.004	-0.05
Fat mass	0.39**	0.49**	0.57**	0.45**	0.38**	0.46**	-0.15	-0.30**

**p<0.01

BMC L2-L4: lumbar bone mineral content

BMC femo: femoral neck bone mineral content

BMD L2-L4: lumbar bone mineral density

BMD femo: femoral neck bone mineral density

Table 4 Pearson Partial Spearman rho correlations between hormone levels and bone parameters controlling for lean body and fat mass

	Estradiol x				Adiponectin x			
	BMC12-14	BMC femo	BMD 12-14	BMD femo	BMC12-14	BMC femo	BMD 12-14	BMD femo
<i>SNP276 G>T</i>								
GG+GT+TT	0.023	0.028	0.035	0.048	0.17*	0.17*	0.1	0.04
GG	0.22	0.10	0.18	0.17	0.34**	0.25*	0.25*	0.06
GT	0.07	0.13	0.13	0.12	0.08	0.1	-0.02	0.03
TT	0.10	0.04	0.19	0.32	0.08	0.66	0.39	0.24
GG+GT	0.17	0.12	0.17	0.16	0.18*	0.16	0.09	0.04
GT+TT	0.012	0.12	0.13	0.13	0.07	0.13	-0.002	0.04
<i>SNP45 T>G</i>								
TT+TG	0.02	0.03	0.04	0.05	0.17*	0.17*	0.10	0.04
TT	0.15	0.11	0.16	0.14	0.21*	0.22**	0.15	0.1
TG	0.78*	0.86**	0.61	0.93**	0.01	0.13	-0.178	-0.25

* P<0.05

**P<0.01

BMC 12-14: lumbar bone mineral content
BMC femo: femoral neck bone mineral content
BMD 12-14: lumbar bone mineral density
BMD femo: femoral neck bone mineral density

GENERAL DISCUSSION AND CONCLUSIONS

The articles presented above report novel findings within their specific research sector.

The first article reports the metabolic signaling relationship between two SIRT1 and AMPK. This study focused specifically on the response of SIRT1 to the α -adrenergic agonist, phenylephrine, in H9c2 cardiac myoblasts, a cell model of cardiac hypertrophy. After 24 and 48 h of phenylephrine treatment, SIRT1 expression and deacetylase activity were significantly increased. SIRT1 upregulation by phenylephrine was not associated to changes in NAD^+ levels, but was blocked by inhibitors of AMP-activated Protein Kinase (AMPK), AraA or STO-609, as well as by siRNA AMPK knockdown. When SIRT1 was inhibited with sirtinol or downregulated by siRNA, H9c2 cell viability was significantly decreased following phenylephrine treatment, showing that SIRT1 improves cell survival under hypertrophic stress. Consequently we have proposed that the increase in SIRT1 activity and expression in H9c2 cells treated with phenylephrine is an adaptive response to the hypertrophic stress, mediated by AMPK, suggesting that adrenergic stimulation of heart cells, activates hypertrophic programming and also promotes a self-protecting and self-regulating mechanisms.

The second article reveals a mechanism that could be involved in the regulation of cardiac hypertrophy by AMPK: we have found that AMPK can modulate the activity of Ornithine decarboxylase (ODC). The upregulation of ODC, the key enzyme in the biosynthesis of polyamines, is a characteristic event in response to agents that induce cardiac hypertrophy. The question is why two apparently contrasting mechanisms are activated at the same time in heart cells committed to hypertrophy. We suspect that AMPK plays a regulatory role in the transition towards the hypertrophic phenotype. The

rapid activation of AMPK following adrenergic stimuli is probably due to the cells effort to guarantee itself energetic substrates in order to respond to an increase in ATP demand. The lack of optimal nutritional conditions (energetic substrates) due to a prolonged activation of AMPK could instead contrast the establishment of hypertrophy, possibly also by means of the negative modulation of ODC activity.

In the Chapter 1 data are provided suggesting that adrenergic stimulation of heart cells along with the hypertrophic program also promotes self-protecting and self-regulating mechanisms. The modulation of ODC activity by AMPK well fits in this model, since it could represent a powerful self-regulatory mechanism.

The other two articles investigated the influence that two single nucleotide polymorphisms (SNP) of the adiponectin gene (SNP +45T/G and +276G/T) have on women's health parameter determinants specifically regarding cardiovascular and bone health. The third article discusses the SNP role in the development of diabetes, because it has been suggested by other studies that the G allele substitution at site 45 increased the risk of its development in the population. Our results suggest that the literature reported increase in the risk of diabetes in subjects that were G allele carriers at site 45 in obese populations when compared to normal weight populations can be caused by a muscle mass difference. A more clear vision of the relationship between SNP and diabetes was possible to disclose because the study was conducted on adolescent girls, a population that is void of many of the other confounding variables present in an adult population.

The fourth article was mainly focused on the prevention of osteoporosis by the studying factors affecting the proper accrual of peak bone mass during adolescence, mainly the interaction between ADIPOQ SNP, estradiol, and bone density. It was found

that the G allele substitution on site 45 favored a greater bone mineral density, which can be explained by three hypothesis. One, that the SNP of ADIPOQ does independently influences the proliferation of osteocytes and thereby bone formation; two, that the SNP of the adiponectin gene influences muscle mass which indirectly influences bone formation; three, is that there is an interaction between the two plausible hypothesis stated prior. We also reported a weak, but present interaction between ADIPOQ SNPs and estradiol and bone density. Following, interpretation of this specific finding will be discussed.

All four studies along with already published findings indicate how SIRT1, AMPK, adiponectin are related to each other, exercise and health. It is already recognized that chronic exercise training causes an increase in SIRT1 and AMPK activity, and adiponectin levels (10,21). Also AMPK is downstream of the adiponectin receptor AdipoR1 and AdipoR2, with a reported preference to AdipoR1 in skeletal muscle (23,27). However, it is of interest to reveal the relationship between these three factors and how they are associated to health and harm.

Adrenergic stimulation is well known to be a key factor in cardiac hypertrophy. Hypertrophic changes occur in response to both alpha and beta adrenergic stimulation in vitro. On a clinical level, down regulation of β -adrenergic receptors in heart failure as a compensatory mechanism elicited to protect the heart from too much sympathetic stimulation, and then becoming desensitized to adrenergic stimulation secondary to the uncoupling of these ARs from their downstream signaling pathways. Again following the reasoning that β -AR signaling is desensitized in heart failure, resulting in the need for higher doses of an agonist to achieve effective increases in myocardial contractility. On the contrary, one of the adaptations to exercise training is the increase in adrenergic

sensitivity. There is an increase in receptor density and a decrease in the amount of epinephrine released at a given work load.

As reported in literature, adrenergic stimulation as well as exercise increases not only SIRT1 activity but also SIRT1 transcription and protein expression (29). This has been consistently found in in-vitro studies as well as in in-vivo studies conducted on humans and mice. This increase in SIRT1 function in response to exercise and adrenergic stimulation is however only seen acutely, followed by an increase in mitochondrial biogenesis marked by the chronic increase of cytochrome C and citrate synthase (29), and markers of proliferation and hypertrophy in-vivo. These biochemical adaptations in response to adrenergic stimulation and exercise seen following the increase in SIRT1 activity are the same seen following the increase in AMPK activity. Suggesting not only a close relationship between both SIRT1 and AMPK, but also that the changes that occur in response to exercise can be explained as a signaling response to the adrenergic stimulation patterns seen during chronic and acute bouts of exercise. Therefore epinephrine release not only causes an immediate responses to cope with increases in demand during exercise, such as increasing heart rate and force, but also signals other cellular changes that have a more chronic effect so to allow a more efficient response to a future increase in demand.

Adiponectin plasma levels is generally viewed as a positive indicator of health. Negatively correlated to fat mass, and increases according to intensity training (30) as well as increasing in response to training independent of fat mass changes (30). We published that the adiponectin gene polymorphism had a novel relationship to lean body mass. Although this is the first time that the adiponectin gene had been linked to muscle mass, previous studies have already reported an increase in muscle cell proliferation in

response to adiponectin (23,31), thereby strengthening the plausible relationship between adiponectin and muscle cell. Administration of adiponectin has also been shown to cause glucose-lowering effects and ameliorate insulin resistance in mice, and adiponectin-deficient mice exhibit insulin resistance and diabetes (32). This insulin-sensitizing effect of adiponectin seems to be mediated by an increase in fatty acid oxidation by the activation of AMP-activated protein kinase (AMPK) and also by peroxisome proliferator-activated receptor α (PPAR α) (32). In contrast, the relationship between Adiponectin and SIRT1 is unclear. Banks et al. (2008) demonstrated that SIRT1 overexpressed mice exhibited beneficial metabolic effects such as increase in insulin sensitivity, resistance to diabetes and hyperadiponectinemia (33). Later, Iwabu et al. (2010) reported that adiponectin exhibited its beneficial effects by causing an increase in SIRT1, thereby concluding that adiponectin regulates the AMPK/SIRT axis (34). The interaction between SIRT1 and adiponectin, as to which is upstream of the other, or if instead there is feedback mechanism, must still be elucidated.

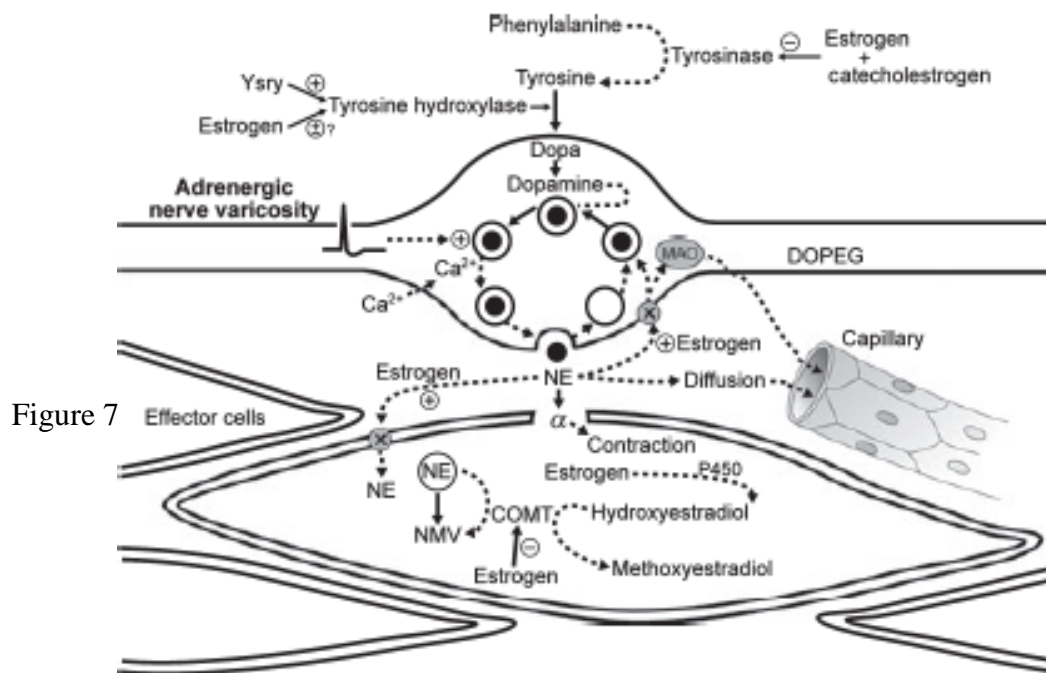
Literature reports a direct relationship between the adrenergic system and adiponectin. In vivo and in vitro studies report that adrenergic agonist significantly decrease adiponectin secretion (35). The decrease in adiponectin secretion in response to adrenergic agonists along with the knowledge of catecholamine secretion in response to chronic and acute exercise explains the increase in adiponectin serum levels after exercise training (decrease in adrenergic basal activity) and the absence changes in adiponectin levels in response to acute exercise (increase in adrenergic activity to meet increase in physiological demand) (36). Concluding that adiponectin secretion is one of the many adaptive responses to exercise mediated by adrenergic signaling and not an immediate response to stress.

Contrary to the accepted belief that high adiponectin levels are an indication of health, it has also been paradoxically reported that high adiponectin levels is correlated with a negative prognosis of survival in chronic heart failure patients (37). It was subsequently found that beta- antagonist treatment in patients reduced adiponectin levels especially in non obese heart failure patients (38) and prognostic value of adiponectin level was severely diminished (39). This paradox can be explained if adiponectin levels are considered as a consequence to the adrenergic system's adaptations during exercise (described above) and a pathological state (high levels of adiponectin). In heart failure patients there is an increase in plasma epinephrine levels, so one would expect a decrease in adiponectin levels, which is, however, the exact opposite of what has been reported. It is also well noted that there is a process of desensitization of the adrenergic response in heart failure patients, so in essence, although there is a lot of circulating epinephrine, since the receptors are not fully functional, it is as if there are low levels of epinephrine, consenting therefore the increase in adiponectin as seen in this specific population. Essentially, adiponectin levels in heart failure patients is not a direct indicator of the patient's prognosis , as proposed by literature, but instead it is an indicator of the functional status of the adrenergic system, which is why treatment with beta blockers reduces the prognosis power of adiponectin levels (39).

After the statement of such a hypothesis, one may ask then, what is the involvement of bone in this relationship, especially when science seems to be so specialized as to view health and the human body as a summation of independent cells, organs, and systems, instead of as a whole. Similar to that above described relationship between, adrenergic system and adiponectin with heart health, the literature reports

findings that can lead to a plausible relationship also between adrenergic system and adiponectin with bone health.

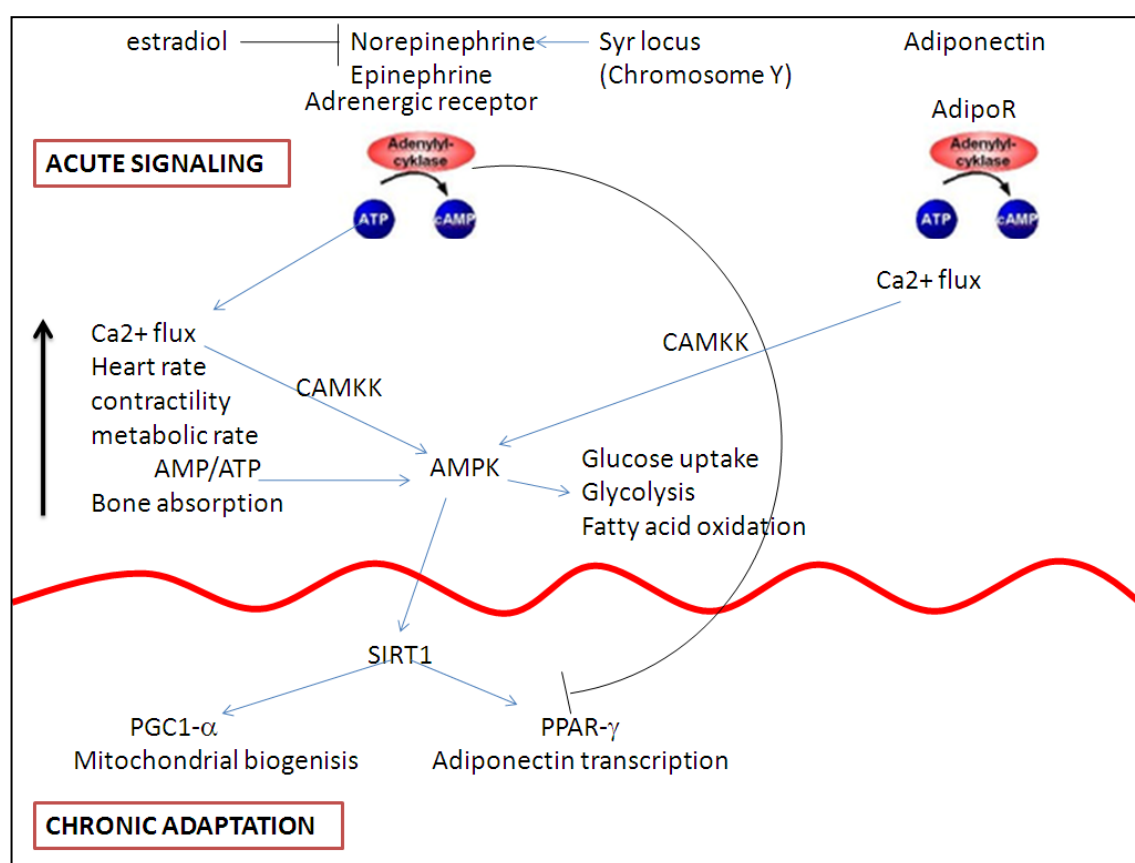
It has been well documented clinically that there is an increase in osteoporosis and cardiovascular disease in females after menopause, at which point there is a decrease in estradiol and adiponectin circulation levels (40,41). A simplistic conclusion that one can form is that there is an increase in disease because there is a decrease in what are recognized as protective hormones in terms of bone health (42-45). However, a broader view of this complex picture is a better fit. Aside from having a decrease in estradiol and adiponectin after menopause, it is also well documented that there is an increase in sympathetic activity (46) , also when compared to males, premenopausal women have a lower sympathetic activity (47). The difference in sympathetic activity between male and females is dependent on the relative sex hormones (47). The Sy locus located on the Y chromosome regulates tyrosine hydroxylase, an enzyme responsible for the production of nor-epinephrine, while 17 β estradiol inhibits tyrosinase activity, another enzyme necessary for the formation of norepinephrine (47) (Figure 7).



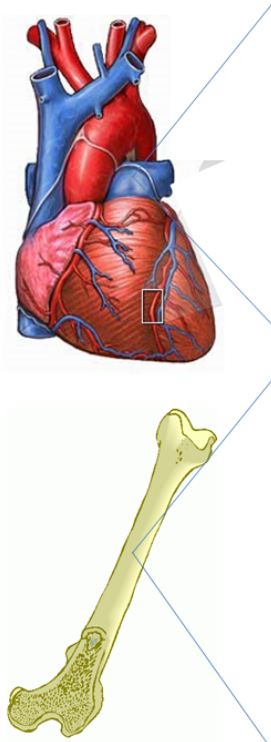
It has also been reported that an effective clinical treatment for osteoporosis is the use of beta antagonists, since adrenergic activity increases absorption of bone (48). Again, as with cardiac health, one can hypothesize that the decrease in adiponectin seen at menopause can be due to the increase in sympathetic activity, since adrenergic stimulation has been reported to significantly decrease adiponectin release (35), and said increase in adrenergic activity is caused by the decrease in estradiol levels (47).

Further, exercise is a well noted method of ensuring bone health and density, by stimulating formation because of osteocytes role as mechano-sensory receptors — regulating the bone's response to stress and mechanical load. Aside from the mechanical effect of exercise training on bone, one can also consider that there are beneficial effects that can be attributed to the fact that training causes a decrease in basal sympathetic activity, explaining why which cycling training (non impact) increase bone density in spinal cord injured patients (49). The decrease sympathetic activity in response to training decreases its inhibitory effect on adiponectin; essentially training

can warrant a similar effect to that of treatment with beta adrenergic antagonists . This hypothesis can also help explain that although adiponectin and estradiol seem to work hand in hand, we found a surprisingly a slight relationship (although significant) between ADIPOQ SNP and estradiol levels and bone density. One can speculate that the missing link between the two hormones is the adrenergic system, a system that is connected to all parts of the body via nervous system (nor-epinephrine) and the circulatory system (epinephrine).



Summary Figure 1



Acute Exercise (during activity)	Chronic Training (basal level)	Heart failure (basal level)
= β AR	\uparrow β AR	\downarrow β AR
\uparrow adrenergic agonist	\downarrow adrenergic agonist	$\uparrow\uparrow$ adrenergic agonist
= adrenergic sensitivity	\uparrow adrenergic sensitivity	\downarrow adrenergic sensitivity
= adiponectin	\uparrow Adiponectin	\uparrow Adiponectin

Acute Exercise (during activity)	Chronic Training (basal level)	Menopause
= β AR	\uparrow β AR	=
\uparrow adrenergic agonist	\downarrow adrenergic agonist	\uparrow adrenergic agonist
= adrenergic sensitivity	\uparrow adrenergic sensitivity	\downarrow adrenergic sensitivity
= adiponectin	\uparrow Adiponectin	\downarrow Adiponectin
= bone density	\uparrow Bone density	\downarrow Bone density

Summary Table 1

Further research towards clarifying the relationship between SIRT1, AMPK, adiponectin, and the adrenergic system is crucial to better understand how to treat and prevent disease. In the future, I would like to specifically investigate the role of calcium within the relationship, since calcium is needed for muscle contraction and cell signaling, and bone is the body's primary calcium reservoir.

REFERENCES TO GENERAL DISCUSSION AND CONCLUSIONS

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